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13. ABSTRACT *(Maximum 200 Words)*

The growth of new blood vessels, or angiogenesis, is an important part of breast cancer biology. Our laboratory has found that a secreted binding protein for fibroblast growth factors (FGF-BP) is expressed in two breast cancer cell lines and primary breast tumor samples. In addition, FGF-BP is expressed in squamous cell carcinoma (SCC) and colon cancer and modulation of FGF-BP expression in these tumor types results in a significant effect on tumor growth and angiogenesis. To obtain a better understanding of the regulation of FGF-BP and how its aberrant expression might lead to activation of angiogenic pathways, we have isolated the human FGF-BP promoter and have determined which functional promoter elements were necessary for its expression. In particular, we have found that the FGF-BP gene is transcriptionally up-regulated by the phorbol ester TPA, and by the epidermal growth factor (EGF), both activating the protein kinase C (PKC) pathway in the ME180 SCC cell line. In the ME180 model, TPA-induced FGF-BP transcription is mediated through a juxtaposed Sp1/AP-1 positive regulatory element, as well as a C/EBP element, in contrast to EGF-induced transcription only being mediated through the AP-1 and C/EBP regulatory elements. Furthermore, the presence of a distinct repressor element was detected whose normal function limits of the response of the promoter to TPA and EGF. Finally, we found that EGF, but not TPA, is able to up-regulate FGF-BP transcription in the MDA-MB-468 breast cancer cell line, mediated through the AP-1 and C/EBP regulatory elements.

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August 1, 1997 to July 31, 2000

P.I.: Benjamin L. Kagan (1999-2000)/ Violaine K. Harris (1997-1999)

Title: A Modulator of FGF's in Breast Cancer

I. Introduction

Locally acting growth factors have many functions, including a pivotal role in inducing the formation of new blood vessels in a healing wound, as well as in a growing tumor. Many studies involving a variety of different approaches have demonstrated that a solid tumor mass cannot grow beyond a few millimeters in size without a sufficient supply of blood to the tumor. Tumor blood vessels provide a pathway for tumor cells to metastasize to distal sites, as well as a source of nourishment (Fidler and Ellis, 1994; Folkman, 1986; Folkman and Klagsburn, 1987; Liotta *et al.*, 1991). It has been reported that in breast cancer a direct correlation exists between blood vessel density in primary tumors and their metastases (Bosari *et al.*, 1992; Horak *et al.*, 1992; Toi *et al.*, 1993; Weidner *et al.*, 1992; Weidner *et al.*, 1991). It is interesting to note that tumor angiogenesis, as reflected in microvessel density, is an independent prognostic indicator in breast cancer patients when tested against other known parameters (e.g. tumor size, estrogen receptor, lymph node status, c-erbB-2 expression).

The most prominent and best studied angiogenesis factors belong to the family of fibroblast growth factor (FGF) polypeptides (Baird and Klagsbrun, 1991; Gospodarowicz *et al.*, 1987). FGF-1 and FGF-2 (aFGF and bFGF, respectively) are unique in that their biological activities can be quenched by binding tightly to heparansulfate proteoglycan molecules in the extracellular matrix (Kiefer *et al.*, 1990; Rogelj *et al.*, 1989; Saksela *et al.*, 1988; Vlodavsky *et al.*, 1987). Two alternate mechanisms of FGF-1 and FGF-2 activation have been established as a result of a multitude of studies over the last decade. One mechanism involves the solubilization of FGF-2 from its storage site by heparanase digestion of the glycosaminoglycan portion of the cell attachment (Bashkin *et al.*, 1989; Moscatelli, 1992; Vlodavsky *et al.*, 1991; Vlodavsky *et al.*, 1988). The second mechanism involves the binding of FGF to a secreted carrier protein delivering the activated FGF to its target receptor. A secreted carrier protein has been described which is able to bind to FGF-1 and FGF-2 in a non-covalent, reversible manner (Wu *et al.*, 1991). FGF-2 bound to this protein was not subject to degradation and retained its mitogenic activity (Wu *et al.*, 1991). This **FGF-binding protein (FGF-BP)** has been studied extensively by our laboratory.

Expression of FGF-BP in cell lines that express FGF-2 results in these cells having a tumorigenic and angiogenic phenotype (Czubayko *et al.*, 1994). FGF-BP transfected cells have been shown to release the protein into their media along with FGF-2 in a non-covalently bound form; the released FGF-2 is now biologically active. *In vivo* growth of FGF-BP positive squamous cell carcinoma (SCC) and colon carcinoma cell lines were inhibited by FGF-BP-targeted ribozyme depletion of endogenous FGF-BP, supporting the idea of an activating step for locally stored FGF-2 resulting from expression and secretion of FGF-BP (Czubayko *et al.*, 1997).

FGF-BP mRNA is expressed in SCC, colon, and breast tumor cell lines and primary tumor tissue (Czubayko *et al.*, 1994). The role of FGF-BP during tumor progression has been studied by our laboratory using skin carcinogenesis as a model for epithelial cancers. We have shown that FGF-BP mRNA is upregulated in the skin during mouse development, but drops to low levels in adult mouse skin. In both mouse and human skin, FGF-BP mRNA and protein levels increase at least 3-fold upon treatment with PKC-activating TPA (12-O-tetradecanoylphorbol-13-acetate), and increase further in DMBA/TPA induced papillomas and carcinomas (Kurtz *et al.*, 1997). **The correlation between FGF-BP expression and tumor promotion by the PKC activator TPA suggests a role for FGF-BP, and its regulation by PKC, in tumorigenesis.**

We have found FGF-BP mRNA to be expressed in two breast cancer cell lines, and 4 out of 6 clinical samples of human breast cancers, by Northern Analysis/Ribonuclease Protection, and RT-PCR, respectively. We have also detected FGF-BP mRNA in the human and mouse mammary gland. **This report summarizes the findings by Violaine Harris and Benjamin Kagan as PIs of the funded research, testing the role of FGF-BP in human breast cancer cell progression and its regulation of expression by protein kinase C (PKC).**

The aims of the grant application were the following:
Aim 1: To study the tumor growth effects of FGF-BP expression in breast cancer cells. and **Aim 2**: To study the mechanisms of regulation of FGF-BP by protein kinase C (PKC).

II. Summary of Results

Figures noted in text below are located in Appendix A.

Isolation and characterization of the human FGF-BP promoter. To study the transcriptional regulation of the human FGF-BP gene, genomic sequences 1.8 kilobases upstream from the 5' UTR sequence of the human FGF-BP cDNA was isolated from a human genomic library. Sequence analysis of the promoter demonstrated the presence of numerous consensus transcription factor binding sites which were conserved between mouse and human FGF-BP promoter sequences and may have functional relevance in FGF-BP regulation. Consensus binding sites included a TATA box (required for transcriptional initiation), a binding site for the CCAAT/enhancer binding protein (C/EBP) family of leucine zipper transcription factors, an AP-1 (Fos/Jun dimers) consensus binding site, and two Sp1 binding sites. The C/EBP transcription factor family plays a central role during inflammation and differentiation (Lekstrom-Himes and Xanthopoulos, 1998). **Figure 1** shows an updated diagram representing positive and negative regulatory elements in the FGF-BP promoter. **The analysis of the FGF-BP promoter was first published in the Journal of Biological Chemistry in July 1998 (Harris *et al.*, 1998), with Violaine Harris (P.I.) as first author.**

Regulation of FGF-BP expression in the ME180 squamous cell carcinoma cell line

TPA regulation of the FGF-BP promoter involves a repressor element juxtaposed to the AP-1 site. Phorbol esters, such as TPA, act as potent tumor promoters through their ability to activate protein kinase C (PKC). We found that FGF-BP gene expression is dramatically upregulated in response to TPA treatment in the ME180 SCC cell line. To study which promoter elements are involved in TPA regulation, a series of promoter deletion mutants was generated and we analyzed the ability of these mutants to drive the expression of a luciferase reporter gene. These analyses revealed that TPA regulation required an interplay between several regulatory elements, including the juxtaposed Sp1(b)/AP-1 site, as well as the C/EBP site. The involvement of these transcription factors in the regulation of FGF-BP was confirmed by electrophoretic mobility shift assay (EMSA). These results demonstrated distinct binding of each of these factors to their respective promoter elements. **The regulation of FGF-BP promoter was published in JBC 1998 (Harris *et al.*, 1998).**

EGF regulation of FGF-BP transcription. Epidermal growth factors (EGF) are potent regulators of cell proliferation and differentiation of many tissue types. The deregulation of the EGF-induced signaling network has been shown to play important roles in the tumorigenesis for several human cancers, including those of the brain, lung, breast, ovary, pancreas, prostate, colon, and squamous cell carcinoma (SCC) of the skin and cervix (Donato *et al.*, 1993; Hynes and Stern, 1994; Salomon *et al.*, 1995; Yuspa, 1994). The EGF family of polypeptide growth factors plays an especially important role in the development of the mammary gland and in the pathogenesis of breast cancer (Kim and Muller, 1999).

We examined the regulation of FGF-BP by EGF in the ME180 SCC cell line, and found that EGF treatment caused a rapid induction of FGF-BP mRNA and transcription. Promoter analysis using the deletion constructs described above revealed that EGF induction was mediated through the AP-1 and C/EBP elements in the FGF-BP promoter. The identity of the factors involved was confirmed by EMSA demonstrating the EGF-induced binding of c-Fos and JunD to the AP-1 site, as well as C/EBPβ and C/EBPδ to the C/EBP site. These results therefore identified transcription factor targets that are important in the regulation of FGF-BP gene expression and the stimulation of angiogenesis by EGF. **The work describing EGF regulation of FGF-BP transcription, with Violaine Harris (P.I.) as first author, and Benjamin Kagan (P.I.) as a co-author, was published in JBC, April 2000 (Harris *et al.*, 2000a).**

Mitogen-activated protein kinase (MAPK) signal transduction pathways which mediate EGF induction of FGF-BP. EGF signaling occurs by binding to the EGF receptor (EGFR or HER1), in a partnership with another EGFR molecule or with other members of the EGFR family (HER2-4 or erbB-2 -3, -4) (Tzahar *et al.*, 1997). HER2 plays a significant role in the progression of breast cancer. Because it is frequently amplified in more aggressive breast cancers, HER2 is currently a target for breast cancer therapy (Earp *et al.*, 1995; Kirschbaum and Yarden, 2000). Auto- or transphosphorylation of the EGFR stimulates a

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number of signal transduction pathways, including the classical MAPK pathway (Ras/Raf/MEK/ERK), which is known to phosphorylate and activate AP-1 transcription factors (Robinson and Cobb, 1997). PKC can mediate this pathway through Ras-dependent or –independent mechanisms (Robinson and Cobb, 1997; Ueda *et al.*, 1996). Other signaling pathways initiated by EGF include the stress-activated protein kinase (SAPK1 or JNK, SAPK2 or p38) (Minden and Karin, 1997), the PI3 kinase (Moghal and Sternberg, 1999), and the JAK/STAT pathways (Ihle, 1996).

To determine which signaling pathways are involved in EGF induction of FGF-BP, we chose to test pharmacological inhibitors of signal transduction components for their effect on FGF-BP regulation. After treatment with the EGFR tyrosine kinase inhibitor Tyrphostin AG1478, we found reduced EGF induction of FGF-BP mRNA. Therefore, the EGFR is essential for the EGF effect on the FGF-BP gene. We have shown previously that TPA induction of FGF-BP transcription was mediated through a PKC-dependent pathway (Harris *et al.*, 1998). To determine whether PKC activation was also required for EGF induction of FGF-BP, we treated ME180 SCC cells with the specific PKC inhibitor Calphostin C (Kobayashi *et al.*, 1989) and observed a complete blockade of EGF induction of FGF-BP mRNA. **This finding demonstrates that PKC activation is central in mediating FGF-BP transcriptional activation upon either EGF or TPA stimulation in ME180 cells.**

To assess the contribution of the MAPK kinases (MEK1/2) to FGF-BP regulation, we tested the effects of pharmacological inhibition of MEK1/2 on EGF signaling. Pretreatment with the drug U0126, which is a potent inhibitor of both MEK1 and MEK2, could effectively block EGF induction of FGF-BP mRNA. Consistent with this result was the observation that expression of dominant negative constructs of MEK blocked EGF induction of the FGF-BP promoter. **These results indicate that selective activation of MEK and ERK is necessary for FGF-BP gene regulation.**

EGF is also known to signal via the PI3 kinase pathway (Moghal and Sternberg, 1999). We used the PI3 kinase inhibitor Wortmannin to test the contribution of PI3 kinase to FGF-BP regulation. Pretreatment with Wortmannin had no effect on EGF induction of FGF-BP mRNA, eliminating a role for PI3 kinase in the regulation of FGF-BP.

Stimulation of the SAPK (JNK and p38) pathway has been shown to regulate AP-1 activity in response to mitogens and stress (Minden and Karin, 1997). Therefore, we tested whether JNK or p38 activation could induce FGF-BP gene expression, by treating with the antibiotic anisomycin. Anisomycin treatment at concentrations below 200 nM is known to be an effective stimulator of both JNK and p38 (Mahadevan and Edwards, 1991). Treatment of ME180 cells with anisomycin alone resulted in a significant and dose-dependent increase in FGF-BP mRNA levels up to 2.3 fold.

We then tested the contribution of p38 to FGF-BP induction using the p38 specific inhibitors SB202190 and SB203850, neither of which inhibit JNK or ERK1/2 (Cuenda *et al.*, 1995). Treatment with either drug along significantly reduced EGF and anisomycin induction of FGF-BP mRNA in dose-dependent manners. The non-inhibitory related compound SB202474 had no effect. Consistent with this observation was that the expression of dominant negative constructs for p38, and not dominant negative JNK, blocked EGF induction of the FGF-BP promoter. **These results demonstrate that both anisomycin and EGF induction of FGF-BP mRNA require p38 activation.**

Overall these date suggest that two important MAPK pathways, MEK/ERK and p38, are necessary for full induction of FGF-BP transcription by EGF. This work has been published in Journal of Biological Chemistry in April 2000 (Harris *et al.*, 2000a).

Identification of a repressor element in the regulation of FGF-BP. There exits a region of low homology between the AP-1 and the C/EBP sites of the human and mouse FGF-BP promoter sequences. This region was not suspected to have any effect on the induction of the promoter by TPA, and therefore an internal deletion removing this region (-57 to -47) was used and tested as a control. Using this Δ57/47 construct surprisingly resulted in TPA induction of the FGF-BP promoter being increased from 7- to 14-fold, suggesting the presence of a possible repressor that might interact at this site. The loss of repression was also observed upon treatment with EGF, with promoter induction increasing from 5- to 8-fold after use with the repressor mutant construct. Deletion of the region between –57 and –47 disrupts an AACGTG (at –60 to –55) that is juxtaposed to the 3' end of the AP-1 site. This sequence shows some similarity to the CACGTG E-box transcription factor consensus sequence that is recognized by a variety of basic helix-loop-helix leucine zipper (bHLHZip) factors (Kadesch, 1993). To test this "non-canonical" E-box for repressor activity, a C to T point mutation was introduced at position –58 into the –118/+62 FGF-BP promoter construct. **Use of the m-58 construct conferred a dramatic increase in TPA induction up to 18-fold above background, and EGF**

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induction up to 10-fold. **This data demonstrates that the point mutation at position –58, as well as the internal deletion from –57 to –47, disrupts repression of the FGF-BP promoter which normally limits the response to TPA and EGF.**

The activity of the repressor element on the FGF-BP promoter was not limited to one cell line. The mutant repressor promoter construct (m-58) showed increased TPA and EGF induction in the BT549 and MCF-7 breast cancer cell lines, as well as the ME180 and HeLa cervical SCC cell lines.

Binding of USF, c-Myc, and Mad2 to the repressor element. To understand the mechanisms of repression through the FGF-BP E-box, we investigated whether we could detect protein-binding complexes using EMSA analysis. We identified binding of a distinct factor to the FGF-BP E-box element that was independent of the AP-1 complex. Mutational analysis convincingly showed that the nucleotides within the E-box element (AACGTG), including the C at position –58, were all required for repressor binding. Using antibodies against different transcription factors within the bHLHZip family, we identified a complex containing USF1 (upstream stimulatory factor) and USF2 binding to the FGF-BP E-box. Binding of USF to this site was induced after TPA or EGF treatment. While EMSA did not reveal any other bHLHZip factor binding, *in vivo* analysis of transcription factor binding was carried out using ChIP assay. Formaldehyde-cross-linked chromatin from TPA-treated ME180 cells was immunoprecipitated with antibodies to c-Myc, Max, Mad-2, and USF1. PCR using primers specific for the FGF-BP promoter was carried out, followed by Southern analysis with an internal primer. ChIP analysis showed c-Myc and Mad-2 binding, as well as USF1 thus confirming the data obtained from EMSA. **USF, as well as c-Myc and Mad-2, binding to the FGF-BP promoter is hypothesized to repress transcriptional induction by TPA and EGF.**

At least two mechanisms exist by which USF binding to the E-box could repress transcriptional induction of the FGF-BP promoter. First, USF could act as a an active repressor through recruitment of co-repressors which interfere with the efficiency of transcription. Secondly, USF binding itself might interfere with the activity of other positive regulatory transcription factors, such as AP-1. The second possibility was tested by generating a number of promoter mutants containing double mutations in the AP-1/repressor(-58) sites or in the C/EBP/repressor(-58) sites, and testing for loss of repression. We were able to show that the promoter construct containing both C/EBP and repressor mutations was still highly inducible (loss of repression), while the double AP-1/repressor mutant was not. This demonstrated that repression through the E-box site was dependent on an intact AP-1 site. The repressor mutation had no effect on AP-1 binding or on the composition of AP-1, suggesting that E-box factor binding somehow interferes with AP-1 transactivating ability. **Therefore, repression through the E-box site is AP-1 dependent.**

Repression of the FGF-BP promoter through methylation of the E-box site. Aberrant methylation is known to be closely associated with cancer progression. Cytosine methylation of CpG dinucleotides is often correlated with repression of genes containing isolated CpG dinucleotides in the regulatory regions of their promoters. Due to the presence of such a CpG dinucleotide at the core of the FGF-BP E-box at position –58, we tested whether methylation of this site could have a repressive effect on FGF-BP transcription. Loss of methylation at this site, in the –58 (C to T) promoter mutant for example, would be an alternative mechanism explaining the enhanced response of this construct to TPA and EGF. *In vitro* methylation of the wild-type FGF-BP promoter construct versus the m-58 promoter construct was conducted so that the methylation pattern of these promoters differed only at the E-box CpG. **When the FGF-BP E-box was methylated, TPA induction was dramatically reduced. This demonstrates that methylation of the repressor site may be a potential mechanism for limiting the transcriptional response to growth factor induction of the FGF-BP gene during cancer progression.**

The work describing repression of the FGF-BP promoter through the E-box site, with Violaine Harris as first author, was accepted in manuscript form in JBC, in June, 2000 (Harris *et al.*, 2000b).

Regulation of FGF-BP expression in the MDA-MB-468 human breast cancer cell line

Detection of endogenous FGF-BP mRNA in MCF-7/ADR and MDA-MB-468 human breast cancer cell lines. Previously, we were able to show that FGF-BP mRNA was expressed in 9 out of 15 breast cancer cell lines, by RT-PCR. To study the regulation of FGF-BP expression in breast cancer cell lines, we wanted to use a quantitative method for detection of FGF-BP mRNA. A ribonuclease protection assay specific for human FGF-BP was developed using a riboprobe derived from a pRC/CMV vector plasmid containing the FGF-BP open reading frame (Czubayko *et al.*, 1994). We were able to detect FGF-BP mRNA only in the MCF-7/ADR

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cell line, an adriamycin resistant clone of the MCF-7 cell line, as well as the ME180 SCC cell line, which was used as a positive control. Northern analysis was also used, screening a wider array of breast cancer cell lines. **We were able to detect expression of FGF-BP mRNA in both MCF-7/ADR and the MDA-MB-468 cell lines. Expression of FGF-BP mRNA, as determined by RNase protection and Northern analysis, is summarized in Table 1.**

EGF, but not TPA, regulation of endogenous FGF-BP in MDA-MB-468 cells. Studies have shown that the MDA-MB-468 cell line overexpresses the EGFR as compared to MCF-7 breast cancer cells (Biscardi *et al.*, 1998; Buick *et al.*, 1990). Biscardi et al. (Biscardi *et al.*, 1998) measure levels of EGFR to be 35 fold that of MCF-7 cells. Because the MDA-MB-468 cell line, like the ME180 cell line, express high levels of the EGFR, we decided to test whether FGF-BP mRNA expressed in these cells can be regulated by EGF and/or TPA. MDA-MB-468 cells were grown to 80% confluency, serum starved for 24 hours, and treated with EGF for 1, 3, 6, or 24 hours. FGF-BP mRNA levels were analyzed by Northern analysis, and we were able to observe that EGF induced FGF-BP upregulation at about 3-fold above control, peaking at 6 hours of EGF treatment (**Figure 2**). The time-course of EGF induction of FGF-BP mRNA in MDA-MB-468 cells was similar to that observed in the ME180 SCC cell line, suggesting similar mechanisms of regulation (Harris *et al.*, 2000a). Because of this similarity, we then tested whether TPA was able to regulate FGF-BP in MDA-MB-468 cells, as seen in ME180 cells. MDA-MB-468 cells were treated with TPA in a similar manner as already published for ME180 cells (Harris *et al.*, 1998). Northern analysis revealed that TPA was not able to effect levels of FGF-BP mRNA expression at any time point tested (**Figure 3**). c-Fos mRNA levels, shown to be a target of TPA through direct activation of PKC (Ron and Kazanietz, 1999), were measured as a positive control for TPA activity. We observed an increase in c-Fos mRNA of about 3 fold at 1 hour after TPA treatment, suggesting that TPA did retain its PKC-inducing activity in the MDA-MB-468 cells. **These data demonstrate that EGF can regulate FGF-BP in MDA-MB-468 cells, in a similar manner to ME180 SCC cells, but not by the phorbol ester TPA, unlike ME180 cells.**

EGF regulation of the FGF-BP promoter in MDA-MB-468 cells. As described above, EGF induces the upregulation of FGF-BP in MDA-MB-468 breast cancer cells. To determine if this regulation occurred at the transcriptional level, we tested whether EGF regulated the activity of FGF-BP promoter in MDA-MB-468 cells. As described above, various portions of the human FGF-BP promoter, full-length, mutated, or deleted, have been cloned upstream of a luciferase reporter gene. These constructs have been used successfully to assess the activity of the FGF-BP promoter in ME180 cells (Harris *et al.*, 2000a; Harris *et al.*, 2000b; Harris *et al.*, 1998). We were able to show that in MDA-MB-468 cells, treatment with EGF was able to induce the activity of the −1060/+62 and −118/+62 promoter constructs 4- to 5-fold above basal (**Figure 4**). Deletion of either the AP-1 or the C/EBP, and not the Sp1(b) site, reduced the induction by EGF of the promoter constructs, suggesting the AP-1 and the C/EBP sites were necessary for EGF induced FGF-BP transcription in this cell line. This observation is similar to what was observed in the ME180 cells (Harris *et al.*, 2000a). Upon further investigation, cell-type specific differences were observed. EGF treatment did not result in the super-induction of the m-58 construct, suggesting that the repressor E-box site was not active in MDA-MB-468 cells. Basal activity of the FGF-BP promoter constructs in MDA-MB-468 cells was also studied. Deletion of the AP-1 site resulted in a statistically significant decrease in promoter basal activity, suggesting the AP-1 site is necessary for basal activity. Deletion of the C/EBP site revealed a statistically significant increase in promoter basal activity, suggesting differences in C/EBP binding to the site affecting both basal and EGF induced activity of the FGF-BP promoter. **These data show that EGF is able to induce the activity of the FGF-BP promoter in MDA-MB-468 cells, through the AP-1 and C/EBP sites, as seen in ME180 cells. Differences lie in the fact that the repressor function of the E-box site is not maintained upon EGF treatment. In addition, C/EBP binding to the FGF-BP promoter may repress basal activity while enhancing promoter activity after EGF treatment.**

The study of FGF-BP regulation in breast cancer cells is being continued by Benjamin Kagan as P.I. of a recently accepted and funded (starting 8/1/2000) pre-doctoral traineeship grant application supported by DOD Breast Cancer Research Program.

The results mentioned above address **technical objective 2**, the mechanisms of regulation of FGF-BP. Because the work addressing the second technical objective took longer than anticipated, in the last six months we have begun to address **technical objective 1**, the study of the tumor growth effects of FGF-BP expression in breast cancer cells. We are generating stable transfected FGF-BP expressing MCF-7 cells using a tetracycline-regulated system, but do not have any data yet to report regarding these cells. Our collaborators, Dr. Kevin

McDonnell and Dr. Anton Wellstein, have been working to develop transgenic mice that express FGF-BP. So far, 90 mice have been generated but all have died during embryogenesis, suggesting a possible role for FGF-BP in development.

III. Key Research Accomplishments

- The human FGF-BP promoter was isolated and cloned revealing positive and negative regulatory elements within a 118 base pair region just upstream of the FGF-BP transcription start site.
- The phorbol ester TPA was shown to upregulate FGF-BP transcription in ME180 squamous cell carcinoma cells. This transcription was mediated through the activation of protein kinase C, and the Sp1, AP-1, and C/EBP positive regulatory elements in the FGF-BP promoter.
- The epidermal growth factor was shown to upregulate FGF-BP mRNA in ME180 SCC cells. Signal transduction was mediated through the EGFR, PKC, MEK/ERK, and p38 pathways. Transcription was mediated through the AP-1 and C/EBP regulatory elements in the promoter.
- A region of the human FGF-BP promoter containing repressor activity was identified to bind the E-box factors USF1, USF2, c-Myc, and Mad2. This repressor region is contained within the –57 to –47 region of the FGF-BP promoter, and is active in TPA and EGF-treated ME180 cells. Methylation of this E-box site also confers the repressor activity.
- Human FGF-BP mRNA was found to be expressed in the MCF-7/ADR, and MDA-MB-468 breast cancer cell lines.
- EGF, but not TPA, is able to upregulate FGF-BP transcription in MDA-MB-468 cells. EGF-induced FGF-BP transcription is mediated through the AP-1 and C/EBP sites of the FGF-BP promoter. The E-box repressor site is not active in these cells.

IV. Reportable Outcomes

Manuscripts, abstracts, and publications produced as a result of this funded research:
1. Harris VK, Liaudet-Coopman ED, Boyle BJ, Wellstein A and Riegel AT, Phorbol ester-induced transcription of a fibroblast growth factor- binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements. *J Biol Chem* **273**(30): 19130-9, 1998.
2. Harris VK, Liaudet-Coopman E, Wellstein A and Riegel AT, A fibroblast growth factor binding protein (FGF-BP) is transcriptionally regulated by phorbol esters and retinoic acid. In: *Proceedings of the American Association for Cancer Research, 89th Annual Meeting, New Orleans, LA,* March 28-April 1,*1998.*
3. Harris VK, Coticchia CM, Kagan BL, Ahmad S, Wellstein A and Riegel AT, Induction of the Angiogenic Modulator Fibroblast Growth Factor-binding Protein by Epidermal Growth Factor Is Mediated through Both MEK/ERK and p38 Signal Transduction Pathways. *J Biol Chem* **275**(15): 10802-10811, 2000.
4. Kagan BL, Harris VK, Coticchia CM, Cabal-Manzano R, Wellstein A and Riegel AT, The role of an angiogenic modulator, the fibroblast growth factor-binding protein, in breast cancer. In: *Era of Hope: The Department of Defense Breast Cancer Research Program Meeting, Atlanta, GA,* June 8-June 11,*2000,* pp. 344.
5. Harris VK, Coticchia CM, List HJ, Wellstein A and Riegel AT, Mitogen-induced expression of the FGF-binding protein is transcriptionally repressed through a non-canonical E-box element. *J Biol Chem,* 2000.

Degrees obtained: Ph.D. in Pharmacology from Georgetown University conferred upon Violaine Harris (P.I) in November of 1998.

Funding applied for based on work supported by this award: A pre-doctoral traineeship supported by the DOD Breast Cancer Research Program was applied for in the 1999 fiscal year, and was awarded to Benjamin Kagan (P.I.). The title of the funded work is **Growth Factor Regulation of an Angiogenic Factor, the Fibroblast Growth Factor-Binding Protein (FGF-BP), in Breast Cancer.**

Employment/research opportunities: Since receiving her Ph.D., Violaine Harris worked for a year in the laboratory of Dr. Anna Tate Riegel at Georgetown University, conducting post-doctoral research funded by the

Susan Komen Breast Cancer Foundation. Dr. Harris has since joined the laboratory of Dr. Stuart Aaronson, for another post-doctoral position, at the Mount Sinai School of Medicine in New York, New York.

V. Conclusions

We have shown that the phorbol ester TPA, and the growth factor EGF, induces FGF-BP transcription in the ME180 SCC cell line. The signal transduction pathways mediating these responses include protein kinase C, and the classical MAPK (MEK/ERK), and p38 pathways. By identifying the pathways involved, we have highlighted several possible targets for potential anti-angiogenic therapy of human cancers, which would utilize FGF-BP's angiogenic properties for tumor growth. We have also demonstrated that an important aspect of FGF-BP transcriptional regulation is the presence of an E-box that mediates AP-1-dependent transcriptional repression. Differences in USF and/or c-Myc binding to the E-box site, along with changes in the methylation status of the promoter, may be important mechanisms by which the extent of repression exerted on the FGF-BP promoter is de-regulated. This would result in an increase in FGF-BP gene transcription and activation of an angiogenic phenotype.

We also were able to observe FGF-BP expression in the MDA-MB-468 cell line. In this model we demonstrated that EGF was able to upregulate FGF-BP transcription. This is important in the context of breast cancer because expression of the EGFR has been inversely correlated with ER expression, and along with expression of the EGFR family member HER2, the EGFR has been correlated with a poor prognosis for breast cancer. FGF-BP expression, and its regulation by EGF in the MDA-MB-468 breast cancer cell line, may suggest that FGF-BP plays a role in the expression of a more angiogenic phenotype in breast cancer.

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VII. Appendices

A. Figures

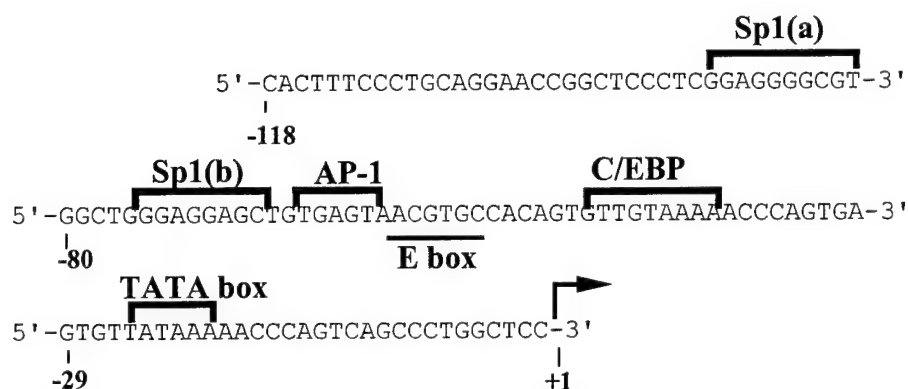**Figure 1. Regulatory region of the FGF-BP promoter**

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                                                              Sp1(a)
                                                         ┌──────────┐
          5'-CACTTTCCCTGCAGGAACCGGCTCCCTCGGAGGGGCGT-3'
                   |
                  -118

              Sp1(b)        AP-1                  C/EBP
           ┌──────────┐ ┌──────────┐          ┌──────────┐
  5'-GGCTGGGAGGAGCTGTGAGTAACGTGCCACAGTGTTGTAAAAACCCAGTGA-3'
        |                    ──────────
       -80                     E box
            TATA box
          ┌──────────┐                      ┌─►
  5'-GTGTTATAAAAACCCAGTCAGCCCTGGCTCC-3'
       |                               |
      -29                             +1
```

Table 1. Levels of FGF-BP, ER, and EGFR in human breast cancer cell lines. (adapted from Biscardi et al., *Mol Carcinog* 21:261-272, 1998)

Cell Line	EGFR	ER	FGF-BP
MCF-7	+	++	-
MCF-7/ADR	+++	-	++
BT20	++	-	-
BT474	-	+	-
T47D	-	+	-
T47Dco	-	-	-
MDA-MB 231	++	-	+/-
MDA-MB 468	++++	-	+++
BT549	++	-	+/-
Hs 578T	++	-	+/-

Figure 2. EGF induction of FGF-BP mRNA in MDA-MB-468 cells. Northern blot (*top panel*), quantification of Northern blot (*bottom panel*).
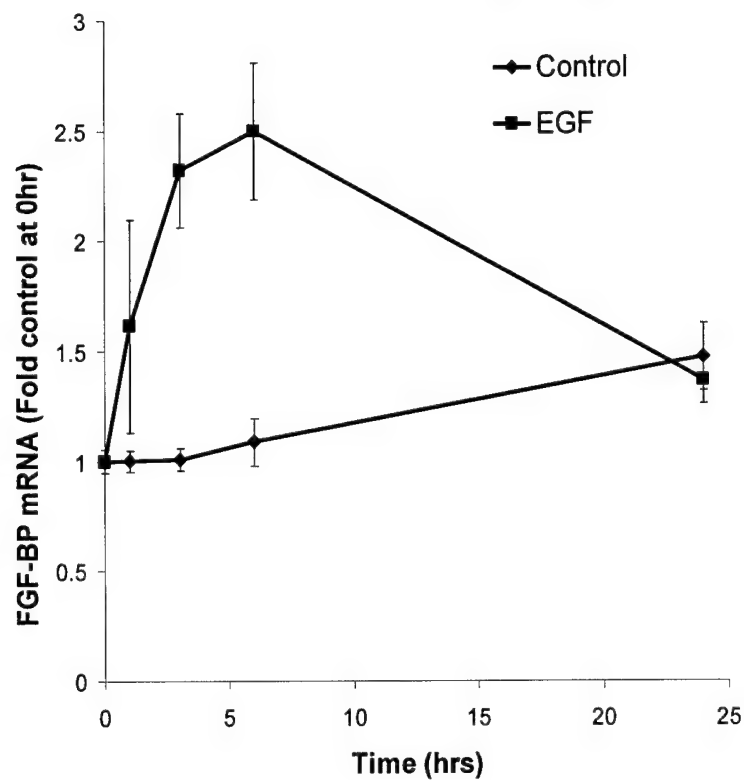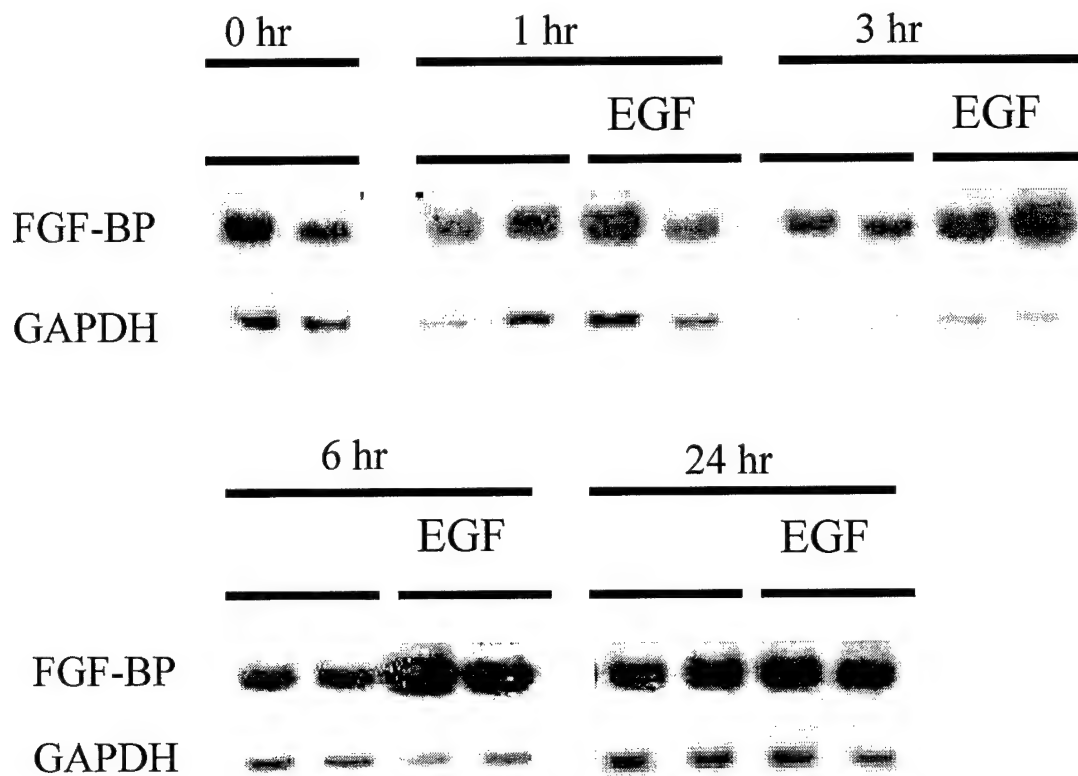
Figure 3. TPA does not induce FGF-BP mRNA in MDA-MB-468 cells, but induces c-fos at 1 hour after treatment.
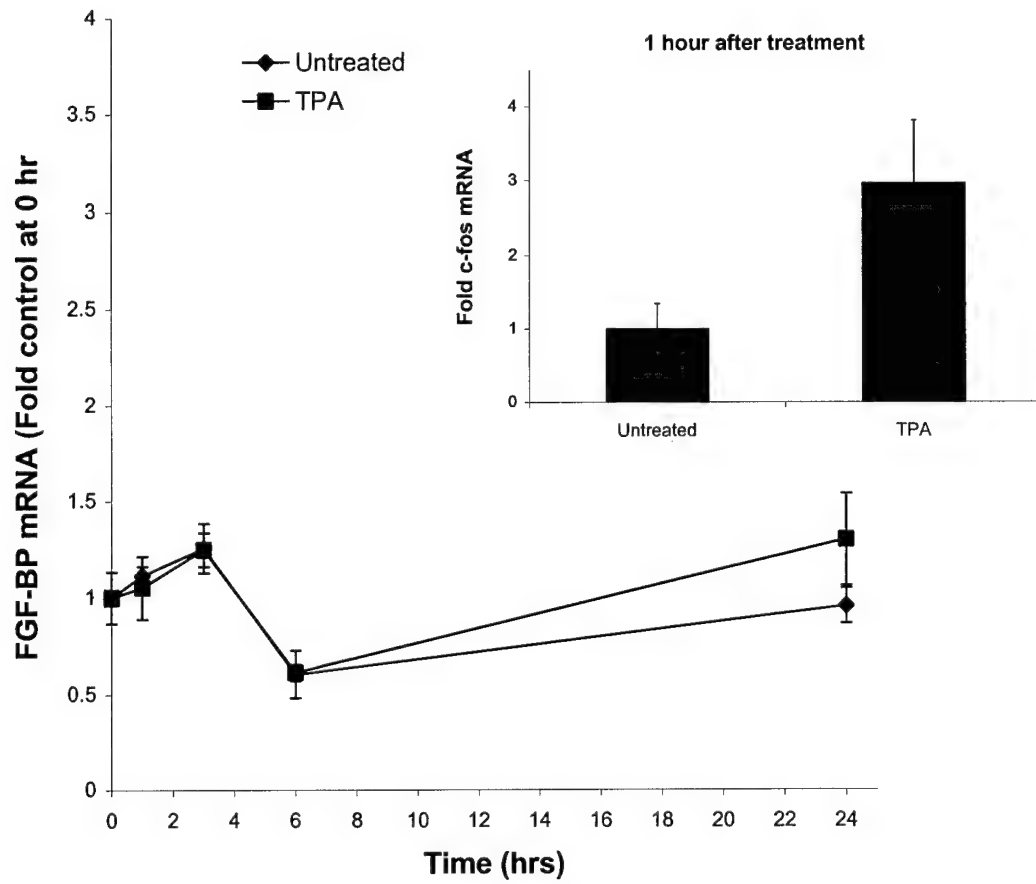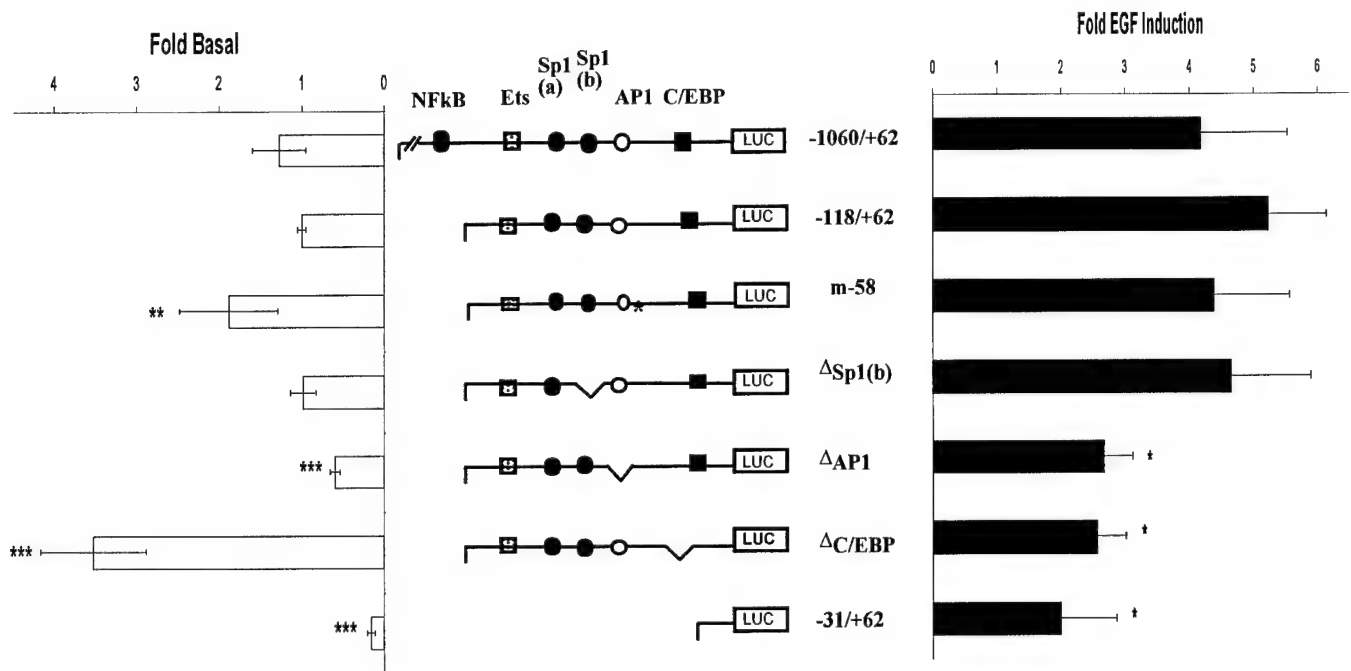
Figure 4. Elements necessary for basal and EGF-inducing activity on the FGF-BP promoter in MDA-MB-468 cells.

B. Reprints obtainable on the world wide web (URLs provided below):

Harris VK, Liaudet-Coopman ED, Boyle BJ, Wellstein A and Riegel AT, Phorbol ester-induced transcription of a fibroblast growth factor- binding protein is modulated by a complex interplay of positive and negative regulatory promoter elements. *J Biol Chem* **273**(30): 19130-9, 1998.
http://www.jbc.org/cgi/reprint/273/30/19130.pdf

Harris VK, Coticchia CM, Kagan BL, Ahmad S, Wellstein A and Riegel AT, Induction of the Angiogenic Modulator Fibroblast Growth Factor-binding Protein by Epidermal Growth Factor Is Mediated through Both MEK/ERK and p38 Signal Transduction Pathways. *J Biol Chem* **275**(15): 10802-10811, 2000.
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http://www.jbc.org/cgi/reprint/M001677200v1.pdf

C. Biographical Sketch

BIOGRAPHICAL SKETCH

Give the following information for the key personnel and consultants and collaborators. Begin with the principal investigator/program director. Photocopy this page for each person.

NAME	POSITION TITLE
Benjamin L. Kagan	Doctoral candidate in Pharmacology

EDUCATION *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Binghamton University- State University of New York, Binghamton, NY	B.S.	1997	Biological Sciences
Georgetown University, Washington, DC	Ph.D. (anticipated)	2002 (anticipated)	Pharmacology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Undergraduate Record:

1993-7   Binghamton University- State University of New York (Final GPA: 3.76), Magna Cum Laude
          GRE- Biological Sciences: 890
          GRE- General: 2030 (760-Analytical, 680-Mathematics, 590-Verbal)

Employment:

1996-7   Binghamton U. Research Scholars Program, research with Anne B. Clark, Ph.D., Department of Biology
1997-   Graduate Student, Dept. of Pharmacology, Lombardi Cancer Center, Georgetown Univ., Wash., DC

Certification & Other Special Scientific Recognition:

2000-   DOD Breast Cancer Research Program Pre-doctoral Traineeship
1997-9   National Institute on Drug Abuse Training Grant/ Dept. of Pharmacology, Georgetown University
1997   Phi Beta Kappa, Magna Cum Laude, B.S. Biology: Binghamton University
1996   Golden Key National Honor Society/ Binghamton University Chapter
1996-7   Binghamton University Research Scholars Program, Binghamton University
1993-1996   Dean's List, Binghamton University

Abstracts and Publications:

Harris VK, Coticchia CM, Kagan BL, Ahmad S, Wellstein A and Riegel AT, Induction of the Angiogenic Modulator Fibroblast Growth Factor-binding Protein by Epidermal Growth Factor Is Mediated through Both MEK/ERK and p38 Signal Transduction Pathways. *J Biol Chem* **275**(15): 10802-10811, 2000.

Kagan BL, Harris VK, Coticchia CM, Cabal-Manzano R, Wellstein A and Riegel AT, The role of an angiogenic modulator, the fibroblast growth factor-binding protein, in breast cancer. In: *Era of Hope: The Department of Defense Breast Cancer Research Program Meeting, Atlanta, GA,* June 8-June 11, *2000,* pp. 344.

Harris VK, Kagan BL, Ray R, Coticchia CM, Liaudet-Coopman EDE, Wellstein A and Riegel AT, Serum induction of the fibroblast growth factor-binding protein (FGF-BP) is mediated through ERK and p38 activation and C/EBP-regulated transcription. (submitted), 2000.

Phorbol Ester-induced Transcription of a Fibroblast Growth Factor-binding Protein Is Modulated by a Complex Interplay of Positive and Negative Regulatory Promoter Elements*

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Earlier studies from our laboratory showed that a secreted binding protein for fibroblast growth factors (FGF-BP) is expressed at high levels in squamous cell carcinoma (SCC) cell lines. Overexpression studies or conversely reduced expression of FGF-BP by ribozyme targeting have elucidated a direct role of this protein in angiogenesis during tumor development. We have also observed a significant up-regulation of FGF-BP during TPA (12-O-tetradecanoylphorbol-13-acetate) promotion of skin cancer. Here we investigate the mechanism of TPA induction of FGF-BP gene expression in the human ME-180 SCC cell line. We found that TPA increased FGF-BP mRNA levels in a time- and dose-dependent manner mediated via the protein kinase C signal transduction pathway. Results from actinomycin D and cycloheximide experiments as well as nuclear transcription assays revealed that TPA up-regulated the steady-state levels of FGF-BP mRNA by increasing its rate of gene transcription independently of *de novo* protein synthesis. We isolated the human FGF-BP promoter and determined by deletion analysis that TPA regulatory elements were all contained in the first 118 base pairs upstream of the transcription start site. Further mutational analysis revealed that full TPA induction required interplay between several regulatory elements with homology to Ets, AP-1, and CAATT/enhancer binding protein C/EBP sites. In addition, deletion or mutation of a 10-base pair region juxtaposed to the AP-1 site dramatically increased TPA induced FGF-BP gene expression. This region represses the extent of the FGF-BP promoter response to TPA and contained sequences recognized by the family of E box helix-loop-helix transcription factors. Gel shift analysis showed specific and TPA-inducible protein binding to the Ets, AP-1, and C/EBP sites. Furthermore, distinct, specific, and TPA-inducible binding to the imperfect E box repressor element was also apparent. Overall, our data indicate that TPA effects on FGF-BP gene transcription are tightly controlled by a complex interplay of positive elements and a novel negative regulatory element.

FGF-BP[1] is a secreted protein that binds to acidic FGF and basic FGF in a non-covalent reversible manner (1). FGF-BP mRNA has been found to be up-regulated in squamous cell carcinoma (SCC) cell lines of different origin, in SCC tumor samples from the head and neck, and in some colon cancer cell lines (1, 2). More recently, developmental expression of the mouse FGF-BP gene was found to be prominent in the skin and intestine during the perinatal phase and is down-regulated in adult mice (3). We previously described that expression of FGF-BP in a non-tumorigenic human cell line (SW-13) which expresses bFGF leads to a tumorigenic and angiogenic phenotype (2). Expression of FGF-BP in these cells solubilizes their endogenous bFGF from its extracellular storage and allows it to reach its receptor, suggesting that FGF-BP serves as an extracellular carrier molecule for bFGF (2, 4). Expression of FGF-BP under the control of a tetracycline-responsive promoter system in SW-13 cells revealed its role during the early phase of tumor growth (5). To assess the significance of FGF-BP endogenously expressed in tumors, we depleted human SCC (ME-180) and colon carcinoma (LS174T) cell lines of their FGF-BP by targeting with specific ribozymes (6). This study showed that the reduction of FGF-BP reduced the release of biologically active bFGF from cells in culture. In addition, the growth and angiogenesis of xenografted tumors in mice was decreased in parallel with the reduction of FGF-BP, suggesting that some human tumors can utilize FGF-BP as an angiogenic switch molecule.

The fact that FGF-BP has been detected in only a few types of tumors, where it seems to play a crucial role in angiogenesis, led us to investigate the mechanisms responsible for turning its expression on or off. Studying the regulation of FGF-BP in SCC cell lines, we showed that all-*trans*-retinoic acid, used as a chemotherapeutic agent against SCCs, down-regulates FGF-BP gene expression *in vitro* by both transcriptional and post-transcriptional mechanisms (7). *In vivo* all-*trans*-retinoic acid treatment reduces FGF-BP expression in SCC xenografts and inhibits their tumor growth and angiogenesis (8). On the other hand, FGF-BP mRNA expression in the adult mouse skin was found to be dramatically increased during the early stages

[1] The abbreviations used are: FGF-BP, fibroblast growth factor-binding protein; bFGF, basic FGF; TPA, 12-O-tetradecanoylphorbol-13-acetate; SCC, squamous cell carcinoma; PKC, protein kinase C; C/EBP, CAATT/enhancer binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IMEM, improved minimum essential medium; kb, kilobase pair(s); DTT, dithiothreitol; PCR, polymerase chain reaction; UTR, untranslated region; CMV, cytomegalovirus.

of 7,12-dimethylbenz[*a*]anthracene/TPA-induced mouse skin papilloma formation (3), as well as in 7,12-dimethylbenz[*a*]anthracene/TPA-treated human skin grafted onto SCID mice.[2] Similarly, FGF-BP expression *in vitro* was up-regulated in epidermal cell lines carrying an activated *ras* gene, implicating the *ras*/PKC pathway in the regulation of FGF-BP (3).

In this context, and given the fact that FGF-BP could play a critical role in the development of human skin cancer, we decided to investigate the effects of the tumor promoter TPA on FGF-BP gene regulation. Our results show that FGF-BP mRNA expression is up-regulated by TPA in the ME-180 SCC cell line and that this induction is mediated by direct transcriptional mechanisms. Analysis of the human FGF-BP promoter reveals that the TPA induction is mediated by cooperation of several inducible regulatory elements. Furthermore, the induction of gene expression by TPA can be modified by a repressor element juxtaposed to the AP-1 site which contains sequences recognized by E box element factors.

MATERIALS AND METHODS

Cell Culture—The ME-180 squamous cell carcinoma cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in improved minimum essential medium (Biofluids Inc., Rockville, MD) with 10% fetal bovine serum (Life Technologies, Inc.).

Northern Analysis—ME-180 cells were grown to 80% confluence on 150-mm tissue culture dishes, washed three times in serum-free IMEM, and then treated 16 h later with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma) in serum-free IMEM. Total RNA was isolated with the RNA STAT-60 method using commercially available reagents and protocols (RNA STAT-60™, Tel-Test, Friendswood, TX). 30 μg of total RNA were separated by electrophoresis in 1.2% formaldehyde-agarose gel and then blotted onto nylon membranes (MSI, Westboro, MA). The blots were prehybridized in 6× SSC (0.9 M sodium chloride, 0.09 M sodium citrate, pH 7.0), 0.5% (w/v) SDS, 5× Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin, 100 μg/ml sonicated salmon sperm DNA) (Life Technologies, Inc.) for 4 h at 42 °C. Hybridization was carried out overnight at 42 °C in the same buffer. After hybridization, blots were washed three times with 2× SSC and 0.1% SDS for 10 min at 42 °C and finally once with 1× SSC and 0.1% SDS for 20 min at 65 °C. Autoradiography was performed using intensifying screens at −70 °C. Blots were stripped by boiling 2 × for 10 min in 1× SSC and 0.1% SDS. Hybridization probes were prepared by random-primed DNA labeling (Amersham Pharmacia Biotech) of purified insert fragments from human FGF-BP (2) and human GAPDH (CLONTECH). The final concentration of the labeled probes was always greater than 10[6] cpm/ml hybridization solution. Quantitation of mRNA levels was performed using a PhosphorImager (Molecular Dynamics).

In Vitro Transcription on Isolated Nuclei—ME-180 cells were grown to 80% confluence on 150-mm tissue culture dishes. Cells were washed three times in serum-free IMEM and then treated 16 h later with TPA in serum-free IMEM for indicated times. Nuclei from 10[7] cells for each time point were isolated after incubation in lysis buffer containing 0.5% Nonidet P-40 as described (7). Nuclear transcription assays were performed with [α-^{32}P]UTP (Amersham Pharmacia Biotech) as described (7). Equal amounts of radioactivity (0.5–1 × 10[7] cpm) were hybridized to nitrocellulose filters containing 3 μg of each plasmid. After hybridization for 4 days at 42 °C, the filters were washed 4 times with 2X SSPE, 0.1% SDS for 5 min at 25 °C and treated for 30 min at 25 °C in 2X SSPE containing 20 μg/ml RNase A. The filters were then washed 4 times for 30 min in 1X SSPE, 1% SDS at 65 °C. The amount of radioactivity present in each slot was determined using a PhosphorImager after overnight exposure, and autoradiograms were exposed for 1–3 days with intensifying screens.

Primer Extension—Primer 1 was designed from the coding region of the human FGF-BP cDNA (5'-GTGAGGCTACAGATCTTC-3'), primer 2 from the FGF-BP 5'-UTR (5'-GTTCACCTTGTTCTGAGCACACG-GATCCA-3'), and a control primer for the 1.2-kb kanamycin RNA (Promega). 10 pmol of each primer was labeled with T4 polynucleotide kinase (Promega) and 30 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech) for 1 h, and labeled primers were purified over a Chro-

maSpin-10 gel filtration column (CLONTECH). Total RNA from ME-180 cells was isolated as described for Northern analysis. ME-180 mRNA was purified from total RNA over an oligo(dT)-cellulose column (Life Technologies, Inc). 100 fmol of each FGF-BP-specific primer was incubated with or without 7 μg of ME-180 mRNA or control primer with or without 2 ng of 1.2-kb kanamycin control RNA (Promega) in the presence of avian myeloblastosis virus reverse transcriptase buffer (50 mM Tris-HCl, 8 mM MgCl$_2$, 30 mM KCl, 1 mM DTT, pH 8.5, Boehringer Mannheim) and allowed to anneal for 1 h at 50 °C. Annealed mixtures were then incubated in the presence of 2 mM dNTPs, 50 units of RNase inhibitor (Boehringer Mannheim), and 40 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) for 1 h at 42 °C. Samples were then run on a 6% polyacrylamide sequencing gel along with radiolabeled *Hin*fI markers and exposed overnight for autoradiography.

Cloning of FGF-BP Gene Promoter—1.8 kb of genomic sequence lying upstream of the human FGF-BP gene was isolated from a human genomic library using the PCR-based PromoterFinder DNA Walking Kit (CLONTECH) according to the manufacturer's recommendations using rTth XL DNA polymerase (Perkin-Elmer). Gene-specific primers derived from the 5'-UTR of the human FGF-BP cDNA were 5'-ACACG-GATCCAGTGCAATCC-3' (+91 to +72) for the primary round of PCR and 5'-GGAGTGAATTGCAGGCTGCAGCTGTGTCAG-3' (+62 to +33) for secondary PCR. Secondary PCR products from a *Dra*I library (1.1 kb) and *Pvu*II library (1.8 kb) were cloned into a TA Cloning Vector pCR2.1 (Invitrogen), sequenced by automated cycle sequencing (ABI PRISM Dye Terminator Cycle Sequencing, Perkin-Elmer), and confirmed to contain contiguous genomic sequence. This sequence has been submitted to GenBank™ (accession number AF062639).

Plasmid Constructs—Promoter fragments were cloned into the PXP1 promoterless luciferase reporter vector (9). PCR products from *Pvu*II (−1829/+62) and *Dra*I (−1060/+62) libraries were removed from pCR2.1 using *Hin*dIII and *Xho*I sites in the pCR2.1 multiple cloning site and ligated into the *Hin*dIII and *Xho*I site of PXP1 to generate pX-1829/+62Luc and pX-1060/+62Luc. Both constructs contained a *Bam*HI site carried over from the pCR2.1 vector and located 3' of the FGF-BP sequence and 5' of the luciferase gene. The 5' promoter deletion constructs pX-118/+62Luc, pX-93/+62Luc, pX-77/+62Luc, pX-67/+62Luc, pX-56/+62Luc, and pX-31/+62Luc were generated by PCR from pX-1060/+62 Luc template using upstream primers spanning −118 to −100, −93 to −76, −77 to −59, −67 to −49, −56 to −35, and −31 to −11, respectively, all containing a 5'-linked *Bam*HI site. Downstream primer was derived from the luciferase gene 5'-CCATCCTCTAGAG-GATAGAATGGCGCCGGGCC-3'. PCR was carried out using *Taq* DNA Polymerase (Boehringer Mannheim). Products were cut with *Bam*HI, gel-purified, and cloned into the PXP1 *Bam*HI site. Correct sequence and orientation were verified by dideoxynucleotide chain termination sequencing with a Sequenase kit 2.0 (U.S. Biochemical).

Internal promoter deletions were generated by PCR-based site-directed mutagenesis. Complementary overlapping oligonucleotides containing specific promoter deletions were generated as follows. Ets site deletion from −76 to −67 was incorporated into primers spanning −58 to −93 and −85 to −47; AP-1 site deletion from −65 to −58 was incorporated into primers spanning −47 to −83 and −72 to −35; Ets/AP-1 site deletion from −76 to −58 was incorporated into primers spanning −49 to −93 and −85 to −36; C/EBPβ site deletion from −47 to −33 was incorporated into primers spanning −24 to −66 and −57 to −11; and deletion from −57 to −47 was incorporated into primers spanning −35 to −75 and −69 to −27. The −58 point mutant was made by incorporating a C to T point mutation at position −58 into primers representing both strands from −70 to −51. For each construct, two separate PCR reactions were carried out with either the *Bam*HI-linked −118 to −99 upstream primer or the luciferase-specific downstream primer. The products were separated from excess primers and mixed, denatured, and allowed to reanneal. Amplification of the heteroduplex with overlapping 3' ends was carried out by 3' extension in the absence of primers followed by amplification using outside primers (*Bam*HI-linked −118 to −99 primer and Luciferase-specific primer) in a secondary round of PCR. Final PCR products were digested with *Bam*HI, gel-purified, and ligated into the PXP1 *Bam*HI site. Correct sequence and orientation were verified by dideoxynucleotide chain termination sequencing with a Sequenase kit 2.0 (U. S. Biochemical Corp.). To control for TPA effects on vector sequences unrelated to the FGF-BP promoter insert, we tested a number of non-TPA responsive promoters inserted into the PXP 1 vector for their response to TPA under the conditions used in our experiments. Control vectors, as seen in Fig. 6, consisted of fragments of the pro-opiomelanocortin promoter (10), the thymidine kinase minimal promoter (9), or the CMV minimal promoter

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[2] A. Aigner and A. Wellstein, unpublished data.

which were cloned into PXP1 vector. All these vectors demonstrated an approximately 2-fold induction after TPA treatment (see "Results").

Transient Transfections and Reporter Gene Assays—24 h before transfection, ME-180 cells were plated in 6-well plates in IMEM, 10% FBS at a density of 750,000 cells/well. For each transfection, 1.0 μg FGF-BP-luciferase construct and 10 μl of LipofectAMINE Reagent (Life Technologies, Inc.) were combined in 200 μl of IMEM, and liposome-DNA complexes were allowed to form at room temperature for 30 min. Volume was increased to 1 ml with IMEM, added to rinsed cells, and incubated for 3 h at 37 °C. Cells were washed and incubated in IMEM for 3 h and then treated for 18 h with vehicle alone (Me$_2$SO, final concentration 0.1%) or 10^{-7} M TPA. Transfection efficiency for each construct was determined by co-transfection with 1.0 ng of a CMV-driven *Renilla* luciferase reporter vector pRL-CMV (Promega) and found to be the same for all BP-PXP1 constructs. However, due to a 2-fold background TPA induction of pRL-CMV (see above), results were normalized for protein content and not for *Renilla* luciferase activity. Cells were lysed by scraping into 150 μl of Passive Lysis buffer (Promega), and cell debris was removed by brief centrifugation. 20 μl of extract was assayed for both firefly and *Renilla* luciferase activity using the Dual-Luciferase™ Reporter assay system (Promega). Light intensity was measured in a Monolight 2010 luminometer. Light units are expressed firefly light units/μg of protein. Protein content of cell extracts was determined by Bradford assay (Bio-Rad).

Gel Shift Assays—ME-180 cells were grown to 80% confluency on 150-mm dishes, serum-starved for 6 h, and treated with or without 10^{-7} M TPA for 90 min. Nuclear extracts were prepared according to Dignam *et al.* (11) with the following modifications. Pelleted cells were resuspended in 1 ml of buffer A (15 mM KCl, 10 mM HEPES, pH 7.6, 2 mM MgCl$_2$, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 1 mM sodium orthovanadate) (12) with 1× Complete™ protease inhibitor mixture (Boehringer Mannheim) and incubated on ice for 10 min. Crude nuclei were pelleted at 700 g and resuspended in 50 μl of ice-cold buffer C (0.42 M NaCl, 20 mM HEPES, pH 7.9, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM sodium orthovanadate, 1× Complete™ protease inhibitor mixture) and vortexed at 4 °C for 15 min. After centrifugation for 10 min at 1000 × g, supernatant was used directly in binding assays and stored at −70 °C.

4.8 pmol of each synthetic double-stranded oligonucleotide was labeled with T4 polynucleotide kinase (Promega) and 50 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech) for 30 min, and labeled primers were purified over a G-25 Sephadex column (Boehringer Mannheim). Binding reactions consisted of 2.5 μg (−80/−63 probe) or 5 μg of ME-180 nuclear extracts, binding buffer (10 mM Tris, pH 7.5, 10 mM KCl, 5% glycerol, 0.1 mM DTT, 0.1 mM EDTA), and 200 ng of poly(dI-dC) (−80/−63 and −70/−51 probes) or 1.0 μg of poly(dA-dT) (−55/−30 probe) and incubated for 10 min on ice. Unlabeled competitor oligonucleotides were added and incubated for another 10 min before adding 20 fmol of labeled probe. Reactions were carried out 45 min on ice and analyzed by 6% polyacrylamide gel electrophoresis in 1× TBE buffer at 80 V. The AP-1 consensus sequence was 5′-CTAGTGATGAGTCAGCC-GGATC-3′.

RESULTS

TPA Increases FGF-BP mRNA in SCCs—We have previously detected an up-regulation of FGF-BP mRNA following TPA treatment of mouse skin during the development of skin tumors (3) and also in human skin xenografts.[2] These data suggest that the control of this angiogenic switch factor may play an important role in skin carcinogenesis. To examine this further we studied the effect of the tumor promoter TPA on FGF-BP gene expression in ME-180 cells which express high levels of the FGF-BP transcript (7). Cells were treated with 10^{-7} M TPA from 1 to 24 h which resulted in an increase in the steady-state levels of FGF-BP mRNA detectable 1 h after treatment (Fig. 1A). PhosphorImager analysis showed that the induction was maximal after 6 h by 452 ± 44% (Fig. 1A). GAPDH mRNA remained unaffected by TPA treatment, as judged relative to the total amount of RNA loaded and was used to standardize FGF-BP mRNA. The dose dependence of TPA induction of FGF-BP mRNA in ME-180 is shown in Fig. 1B. We estimated the half-maximal effective concentration as 1 nM. The inductive effect of TPA on FGF-BP mRNA was also observed in two other SCC cell lines, FaDu and A431 (data not shown) demonstrating that TPA induction of FGF-BP mRNA is generally preserved in SCC cell lines.

To establish whether TPA induction of FGF-BP mRNA was mediated through a PKC-dependent pathway, ME-180 cells were pretreated or not pretreated for 2 h with 100 nM highly specific PKC inhibitor, calphostin C (13), and then treated with or without 10^{-7} M TPA for 4 h. As can be seen in Fig. 1C, pretreatment of the cells with calphostin C prior to TPA treatment totally blocked the TPA effect, demonstrating that the induction of FGF-BP transcript by TPA is mediated via a PKC-dependent mechanism. In addition, it is known that TPA causes an immediate up-regulation of PKC followed by long term down-regulation of PKC activity. In contrast, although long term calphostin C appears to be able to down-regulate protein kinase C, it does not cause early induction of PKC activation but rather blocks the inductive effects of TPA on PKC (14). Thus the fact that Calphostin C causes no induction of FGF-BP mRNA and blocks the TPA effect argues that induction of FGF-BP mRNA is through an up-regulation of PKC activity rather than a consequence of long term down-regulation.

Mechanism of TPA Induction of FGF-BP mRNA—We have previously shown that FGF-BP gene expression can be regulated through both transcriptional and post-transcriptional mechanisms (7). Therefore we next attempted to determine whether the TPA induction of FGF-BP mRNA was at the transcriptional or post-transcriptional level. We first assessed whether TPA treatment affected the stability of the FGF-BP mRNA. Experiments were performed to determine whether addition of inhibitors of transcription (actinomycin D) or translation (cycloheximide) could inhibit the TPA induction of FGF-BP mRNA. Actinomycin D (5 μg/ml) or cycloheximide (10 μg/ml) were added with or without TPA (10^{-7} M), and FGF-BP mRNA levels were determined 6 h after treatment. As shown in Fig. 2A, simultaneous addition of TPA and actinomycin D completely blocked the TPA induction, whereas simultaneous addition of TPA and cycloheximide had no effect. These data suggest that TPA directly increased the rate of FGF-BP gene transcription independently of *de novo* protein synthesis and did not affect the stability of the FGF-BP transcript. To verify further that the stability of the FGF-BP transcript was not modified by TPA treatment, ME-180 cells were pretreated for 2 h with TPA and then actinomycin D was added to inhibit transcription. As shown in Fig. 2B, pretreatment of cells with TPA did not increase the half-life of the FGF-BP mRNA indicating that the stability of the FGF-BP transcript is not affected by TPA.

To prove directly that TPA increases the rate of transcription of the FGF-BP gene, we then performed nuclear transcription run-on assays. ME-180 cells were treated with or without 10^{-7} M TPA for various periods. ^{32}P-Labeled nascent transcripts were prepared from isolated ME-180 cell nuclei and hybridized to a nylon membrane bearing immobilized target DNA sequences. As shown in Fig. 3, TPA increased FGF-BP transcript levels maximally after 1 h of treatment. Quantitation and normalization to β-actin showed that FGF-BP transcription was up-regulated by 647 ± 1, 448 ± 16, and 197 ± 31% after 1, 4, and 24 h of treatment, respectively (Fig. 3). β-Actin plasmid DNA was used as a control since transcription of this gene remained constant. These findings are consistent with the above results studying steady-state mRNA and the effects of actinomycin D and cycloheximide treatment. Clearly the induction of FGF-BP mRNA by TPA in ME-180 cells is directly due to a rapid up-regulation of transcription.

Isolation and Characterization of the Human FGF-BP Promoter—In order to understand better the transcriptional reg-
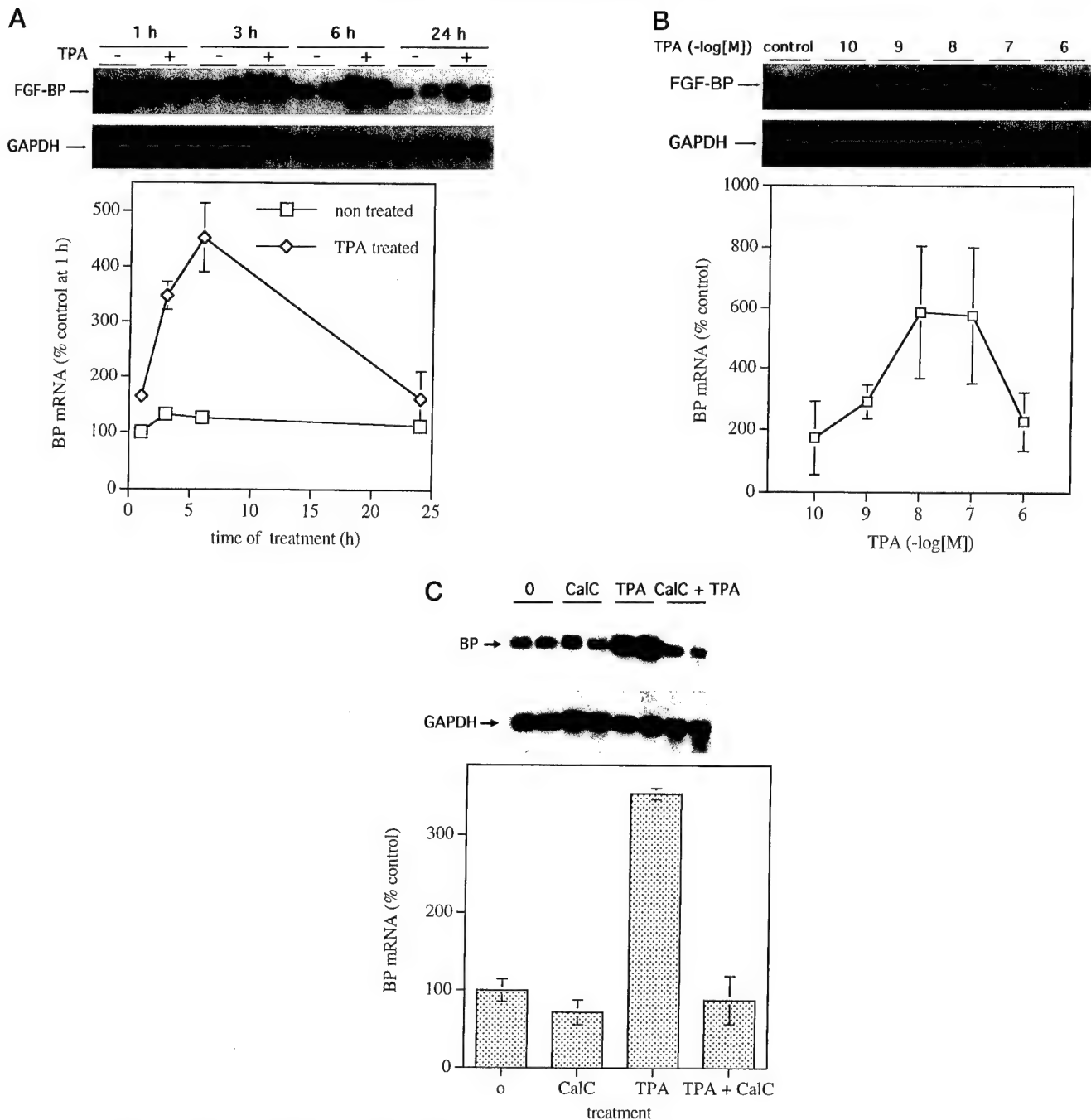
A

B

C

FIG. 1. **TPA effect on FGF-BP mRNA in the ME-180 SCC cell line.** The respective *upper panels* for (*A–C*) are representative Northern blot analyses performed with 30 μg of total RNA/lane as described under "Materials and Methods." Bands corresponding to FGF-BP mRNA (1.2 kb) and the control gene GAPDH mRNA (1.3 kb) are indicated. The respective *lower panels* (*A–C*) show results from quantitation of Northern blots. Signal intensities were quantified by phosphorimaging and normalized to the control gene, GAPDH. The mean ± S.D. of two separate experiments is given. *A*, time dependence of FGF-BP mRNA induction after exposure to 10^{-7} M TPA. *B*, concentration dependence of FGF-BP mRNA after 6 h induction by TPA. *C*, the effect of calphostin C on the TPA induction of FGF-BP mRNA. ME-180 cells were pretreated or not pretreated for 2 h with 10^{-7} M calphostin C (*CalC*) and then treated for 4 h with or without 10^{-7} M TPA in absence or presence of calphostin C.

ulation of the human FGF-BP gene, 1.8 kb of genomic sequence upstream to the known 5′-UTR sequence of human FGF-BP cDNA was isolated from a human genomic library and sequenced. The transcription start site of the human gene was determined using primer extension analysis with nested primers derived from known cDNA sequence (Fig. 4). The precise start site compatible with the primer extension results is indicated in Fig. 5. Alignment between the human and mouse FGF-BP promoter which we cloned previously (3) revealed a region of high homology with 70% nucleotide identity within the first 200 nucleotides upstream from the transcription start

(Fig. 5). Nucleotide homology dropped significantly in more upstream sequences, suggesting that the proximal conserved 200 nucleotides of the promoter could be important for transcriptional regulation of FGF-BP in both species.

Sequence analysis of the promoter demonstrated the presence of numerous consensus transcription factor binding sites that were conserved between mouse and human FGF-BP promoters and that may have functional importance in FGF-BP regulation. As shown in Fig. 5, a consensus TATA box is located at about −25 base pairs upstream from the transcription start for both promoters. Between −48 and −40 of the human
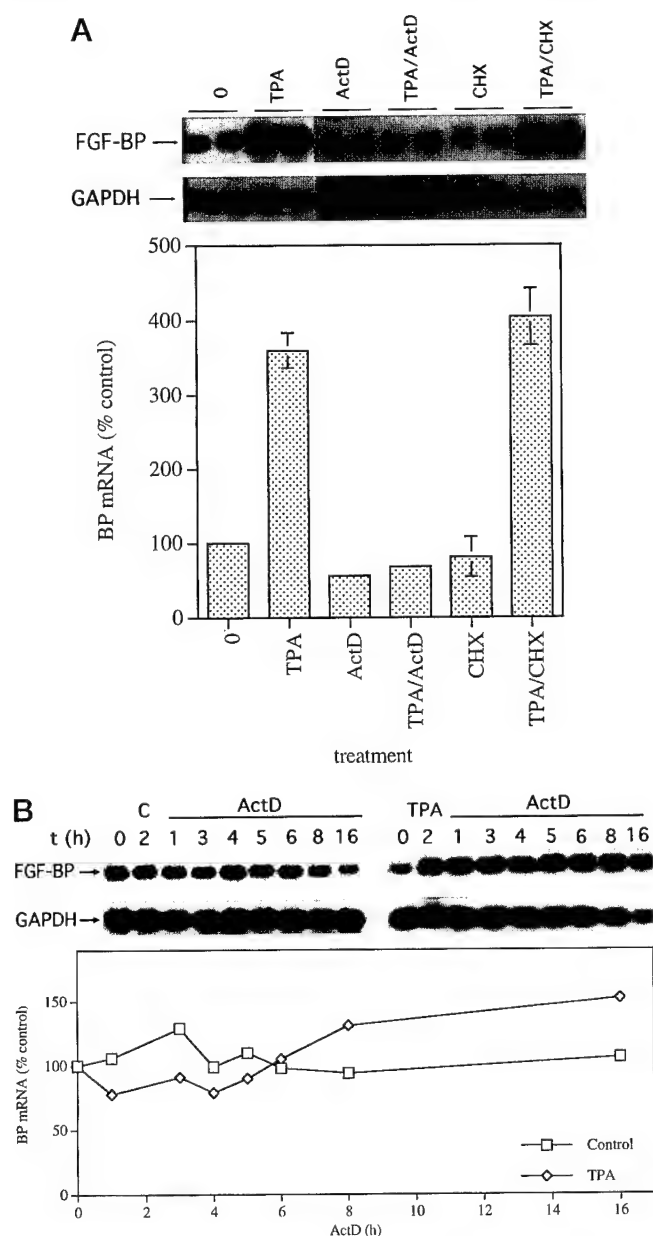
A

FIG. 3. **Transcription run-on analysis of the effects of TPA on FGF-BP gene expression.** Nuclei were isolated from subconfluent layers of ME-180 cells treated or not with 10^{-7} M TPA for 1, 4, or 24 h, and nascent RNA was extended *in vitro* as described under "Materials and Methods." Labeled RNAs (10^7 cpm) were hybridized to nylon membranes on which 3 μg of the pRC/FGF-BP vector was immobilized. β-Actin was used as an internal control and pRC/CMV as a background control vector. Signal intensities were quantified by phosphorimaging and normalized to the control gene β-actin. Hybridization data are shown in the *upper panel* and quantitative data (mean ± S.D.) derived from two independent experiments in the *lower panel*.

B

FIG. 2. **Mechanism of TPA induction of FGF-BP mRNA levels.** The respective *upper panels* of A and B are representative Northern blot analyses performed as described in the legend to Fig. 1. The respective *lower panels* of A and B represent quantification of data in *upper panels*. Signal intensities were quantified by phosphorimaging and normalized to GAPDH. Results represent mean ± S.D. of two independent experiments. *A*, effect of actinomycin D and cycloheximide on the FGF-BP mRNA induction by TPA. ME-180 cells were treated for 6 h in the absence or presence of 10^{-7} M TPA in combination with 5 μg/ml actinomycin D (*ActD*) or 10 μg/ml cycloheximide (*CHX*). *B*, analysis of turnover of FGF-BP mRNA in TPA-treated cells. ME-180 cells were treated with vehicle alone or 10^{-7} M TPA for 2 h, and 5 μg/ml actinomycin D was then added to control and to TPA-treated cells for 0–16 h. Total RNA was isolated and hybridized sequentially with FGF-BP and GAPDH probes as described in Fig. 1.

FGF-BP promoter we found a highly conserved consensus binding site for C/EBPβ, a member of the CCAAT/enhancer binding protein (C/EBP) family of leucine zipper transcription factors which play a central role in the acute phase response and in a number of cell differentiation pathways (15–17). An AP-1 consensus binding site (−65 to −59) lies juxtaposed to a sequence with homology to an Ets factor binding motif (−76 to −68), suggesting potential functional similarity to the juxtaposed Ets/AP-1 site found in the polyoma virus enhancer and in the
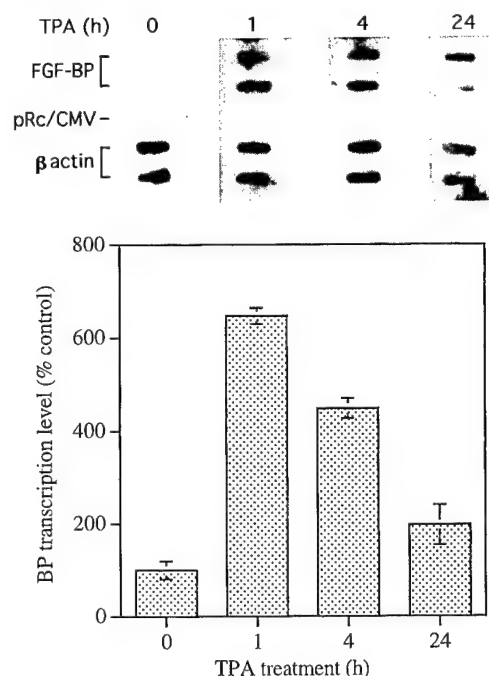
collagenase promoter (18, 19). In addition, a consensus Sp1 factor binding site (−90 to −80), an additional Ets factor binding motif (−107 to −100), and a potential NF-κB-binding site (−185 to −176) are located in the conserved region of the promoter and may play a role in transcriptional regulation of FGF-BP as well.

Functional Analysis of the Human FGF-BP Promoter—To identify the functional promoter elements involved in FGF-BP gene regulation by TPA, progressive 5′ deletion mutants were constructed based on the location of consensus factor binding sites on the promoter. Deletion constructs were transiently transfected into ME-180 cells, and their relative luciferase activity was assayed in the absence or presence of TPA (Fig. 6). The basal activity of each vector is shown in the *left panel* of Figs. 6 and 7. The empty PXP1 vector had no detectable luciferase activity either in the absence or presence of TPA (data not shown). However, we did observe a background, approximately 2-fold, TPA induction of the PXP1 vector when several unrelated minimal promoters (*i.e.* thymidine kinase minimal promoter, the CMV minimal promoter, and the pro-opiomelanocortin minimal promoter) were treated with TPA after transfection into ME-180 cells (Fig. 6, *control vector*). Background induction by TPA of a variety of vectors has been described previously and is presumably mediated through cryptic sites in the PXP1 plasmid (20). The TPA induction due to the inserted FGF-BP promoter was considered to be that observed above the background control vector induction. The FGF-BP promoter from −1060 to +62 was induced about 4-fold above control vector in the presence of TPA and showed the same TPA inducibility as the full-length 1.8-kb promoter construct (data not shown). Deletion from −1060 to −118, which removed 950 base pairs of promoter sequence including the potential NF-κB
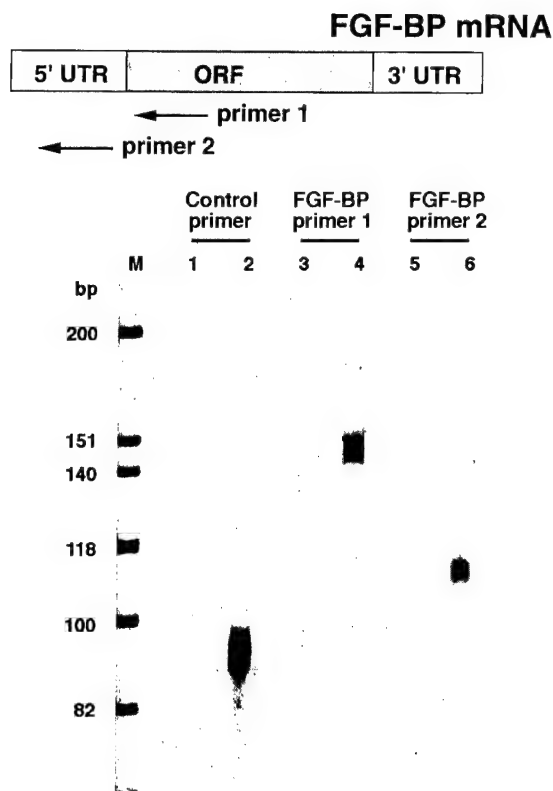
FGF-BP mRNA

FIG. 4. **Mapping of the FGF-BP transcription start site.** Primer extension of the FGF-BP transcript. *Upper panel* shows location of primers derived from the coding region (FGF-BP primer 1, *lower panel, lanes 3* and *4*) and 5'-UTR (FGF-BP primer 2, *lower panel, lanes 5* and *6*) of the human FGF-BP cDNA. Extension reaction was carried out in the absence (*lanes 3* and *5*) or presence (*lanes 4* and *6*) of mRNA isolated from ME-180 cells. A control primer was used in the absence (*lane 1*) or presence (*lane 2*) of 1.2-kb kanamycin control RNA. The transcription start site derived from these results is shown as an *asterisk* in Fig. 5.

site, had no effect on TPA induction and was also induced 5-fold above background (Fig. 6). Similarly, deletion from −118 to −93, which removed one of the potential Ets-binding sites, retained full TPA induction. Removal of the consensus Sp1 binding site from −93 to −77 had no effect on TPA induction of the FGF-BP promoter. However, the 5' deletion to −77 caused an 80% decrease in basal activity of the promoter (Fig. 6, *left panel*) suggesting that the Sp1 consensus site is a predominant mediator of basal promoter activity of FGF-BP but is not required for TPA induction.

TPA induction was significantly reduced upon deletion of the potential Ets factor binding site from −77 to −67 and is reduced even further upon deletion of the AP-1 site from −67 to −56 (Fig. 6), indicating that each of these sites contributes to some degree in TPA induction. The basal activity of these constructs was similar at about 10–20% of the −118 construct. Finally, deletion from −56 to −31, which removes sequences containing homology to a C/EBPβ-binding site, abolished any remaining TPA induction to the background control vector level. The basal activity of this vector was 5% that of the −118/+62 vector.

Contribution of Ets, AP-1, and C/EBPβ Sites to TPA Induction—In order to better understand the contribution of each individual consensus binding site to TPA induction, internal promoter deletions were introduced and tested for TPA inducibility within the context of the promoter from −118 to +62. Deletion of the Ets site alone (−76 to −67) or deletion of the AP-1 site alone (−65 to −58) reduced TPA induction slightly to the intact promoter (Fig. 7). Deletion of both Ets and AP-1 (−76 to −58), however, resulted in a significant decrease in both

basal activity and in TPA induction, indicating that both sites act in cooperation for full promoter activity. However, loss of the juxtaposed Ets/AP-1 site does not completely abolish TPA induction, suggesting that additional sites are also involved.

The contribution of the C/EBPβ binding motif to TPA induction of the FGF-BP promoter was determined by an internal deletion from −47 to −33 (Fig. 7, ΔC/EBPβ). Consistent with the 5' deletion construct which contained only the C/EBPβ site and retained some TPA inducibility (Fig. 6, −56/+62), an internal deletion of this site showed a significant decrease in TPA induction. Activation of C/EBPβ has been shown to occur through *ras*-dependent phosphorylation and is involved in phorbol ester induction of genes such as MDR1 (21–23). Similarly, C/EBPβ seems to play a role in TPA induction of the FGF-BP promoter since deletion of this site reduces the overall induction by TPA.

TPA Regulation of the FGF-BP Promoter Involves a Repressor Element Juxtaposed to the AP-1 Site—Between the AP-1 site and the C/EBPβ site lies a region of low homology between the human and mouse EGF-BP promoter sequences. Because this region was not suspected to have any effect on TPA induction, an internal deletion removing this region (−57 to −47) was tested as a control. Surprisingly, in the Δ57/47 construct, TPA induction of the FGF-BP promoter increased from approximately 5 to 11-fold, suggesting the presence of a possible repressor which may interact with this site. The lack of sequence conservation between the human and mouse in this region may reflect a difference in the regulation of FGF-BP between the two species. The −57 to −47 deletion disrupts an AACGTG (−60 to −55) which is juxtaposed to the 3' end of the AP-1 site and which shows some similarity to the CACGTG E box element recognized by a number of helix-loop-helix factors (24). To test this imperfect E box for repressor activity, a C to T point mutation at position −58 was introduced into the −118/+62 BP promoter construct (Fig. 7). This mutant-58 (m-58) construct showed a dramatic increase in TPA induction up to 16-fold above background. Moreover, when the −58 point mutation is introduced into the ΔC/EBPβ construct (Fig. 7, ΔC/EBPβ/m-58), this promoter mutant also showed increased fold induction by TPA, suggesting that repression mediated by this site is not dependent on the C/EBPβ site. These data show that the point mutation at position −58, as well as the internal deletion from −57 to −47, disrupts repression of the FGF-BP promoter which normally limits the response to TPA.

Transcription Factor Binding to FGF-BP Promoter Elements—In order to ascertain that TPA induction of FGF-BP was due to direct activation by transcription factors, we performed gel retardation analysis to show transcription factor binding to FGF-BP promoter elements. By using labeled promoter sequence from −80 to −63 containing the putative Ets-binding site as a probe (Fig. 8A), the binding of three specific protein complexes in the presence of ME-180 nuclear extracts was detected (Fig. 8B). Protein binding to all three complexes was increased in the presence of TPA (Fig. 8B, *lane 3*) and was specifically competed away in the presence of excess unlabeled −80/−63 oligonucleotides (*lanes 4* and *5*). Further competition analysis showed that the factors binding to the −80/−63 element were only weakly competed by consensus Ets elements from the collagenase promoter (25) and polyoma virus enhancer (26), requiring over 100-fold excess in order to compete for binding (data not shown). It has previously been described that specific residues flanking the GGA trinucleotide motif of the Ets site are required for high affinity sequence-specific binding of individual Ets family members (27–29). Therefore, our data suggest that the −80/−63 element on the FGF-BP

+1

-1829   -1060   -250   +62

-250
Human FGF-BP TCTCCCCATCAAGGAGGGTCATTGTGAGGGTGAGTTGTCCTC....CT......ATCCTGGCCACAGGGT
            || | | | | | | | | | | | ||||| || | | ||| | ||| |
Mouse FGF-BP AGAGAATATGGAAAGGCACTAGAGGAAATGGGCCCTTTCCTCTGTTCTTACAGAAAGATCGCTGCTGGAT
-265

GGACAGGGAAGGGCCT....AGAGGAAGCGTTTGATGGCATGCAGGTAACCCCCCTTTTCACTGGATTAC
|| | ||||||||| | ||||| | || | ||||| | |||||| || |||||| |
GGGAAAGGAAGGGCTTCCAAGGAGGAGGGCTTGCAGCAAATGCA...ACCCCCCCCAGCCATTGGATTCC
-118 NF-kB

ATCCAGCACTTTCCCTGCAGGAACCGGCTCCCTCGGAGGGGCGTGGCTGGGAGGAGCTGTGAGTAACGTG
| |||| || ||||||| ||||| | |||||| | ||||| |||| ||||||||| | |||| ||| ||
ACCCAGTACCTTCCCTCCAGGA.CTGGCTCTCCCTGAGGGGTGTGGCTGGGAGGAGC.GTGAATAAAGT.
            Ets -31   -25   SP1   Ets   AP1
                                                                        *
CCACAGTGTTGTAAAAACC.CAGTGAG....TGTTATAAAAACCCAGT.CAGCCTG.GCTCCTG.TTGAA
|| |||||||||||| || | | ||||| || ||||||| ||| || ||||| | ||| ||| |||
.ATGTGAGTTGTAAAAGCCACCTTGAGCTCCTG.TATAAA....TAGTACAACCTGCACCCCAGCGTGAG
        C/EBPβ   TATA box   *
                                                        +62

TAGTCTACCCCCCTTGCACTCTACCTGACACAGCTGCAGCCTGCAATTCACTCC
|| | | || |||| ||| || || ||| ||| | |||||
GACTGGAAGCC...TGCATTC..CCCAGTACACCTGGA.TCTGCACACTCACAG
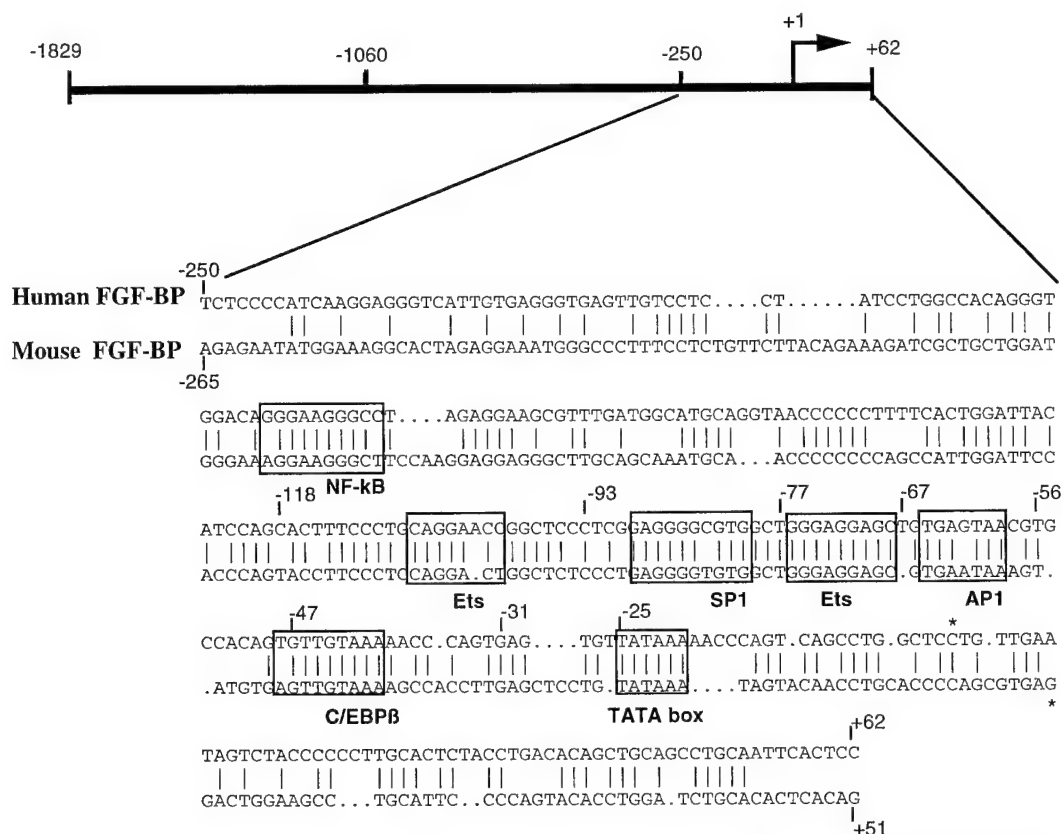   +51

FIG. 5. **Structure and homology of the human FGF-BP promoter.** *Top*, the structure of the human FGF-BP promoter from −1829 to +62 is shown schematically. *Bottom*, nucleotide homology between promoter sequences of human (*top strand*) and mouse (*bottom strand*) FGF-BP. Transcription start sites are indicated by an *asterisk*. *Vertical lines* indicate homologous nucleotides between the mouse and human, and *dotted lines* represent gaps in the homology. Consensus transcription factor binding sites are *boxed*. *Numbers* on the *top strand* correspond to the numbering of the human FGF-BP promoter and show the location of the distal end points used to create the luciferase promoter constructs in Fig. 6.
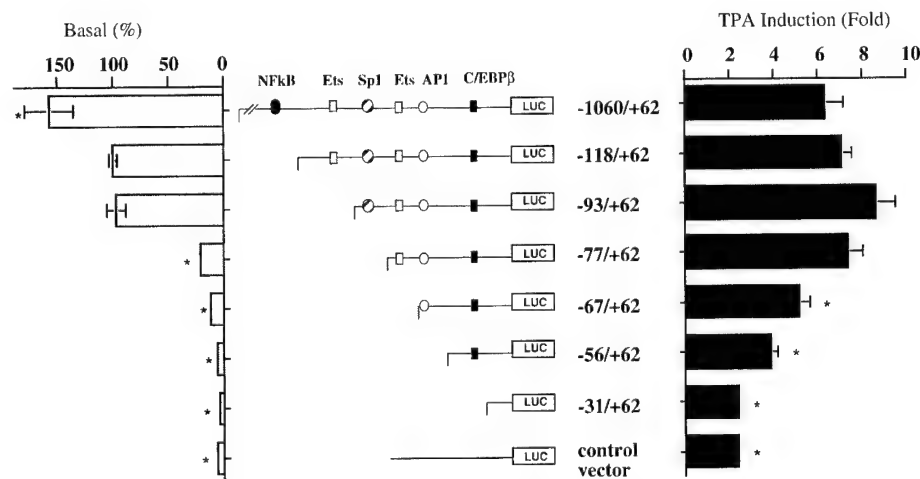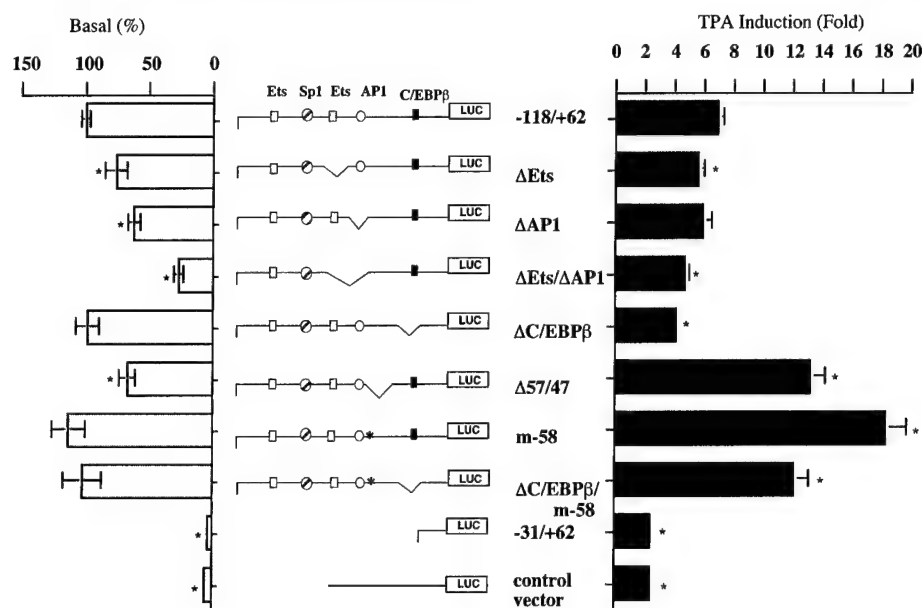
Basal (%)   TPA Induction (Fold)

150 100 50 0   NFkB Ets Sp1 Ets AP1 C/EBPβ   0 2 4 6 8 10

-1060/+62

-118/+62

-93/+62

-77/+62

-67/+62

-56/+62

-31/+62

control
vector

FIG. 6. **Effect of progressive 5′ deletions on basal activity and TPA induction of FGF-BP promoter.** The *left* histogram indicates the impact of the promoter deletion on the basal activity of each construct. The basal activity of the −118/+62 construct was set at 100%. The control vector shown is the thymidine kinase minimal promoter in PXP1. The *right* histogram shows the transcriptional activity in the presence of TPA 10^{-7} M for each FGF-BP promoter deletion construct (*center*). Each promoter construct was transiently transfected into ME-180 cells, and luciferase activity is expressed as fold induction of TPA-treated over untreated for each construct. The mean basal activity of the −118/+62 construct was 15,000 light units per µg of protein and the mean TPA-induced level was 100,000 light units/µg of protein. Values represent the mean ± S.E. from at least three separate experiments, each done in triplicate wells. *Asterisk* indicates significant difference ($p < 0.05$) from the −118/+62 promoter construct.

promoter could bind an Ets family member other than the Ets-1 or Ets-2 proto-oncogenes (26).

To determine transcription factor binding to the C/EBPβ site, gel shift analysis was carried out using labeled promoter sequence from −55 to −30 (Fig. 8A) as a probe. In the presence of ME-180 extracts, the −55/−30 element bound one predominant complex (Fig. 8C), which demonstrated increased binding in the presence of TPA (*lane 3*). The majority of the complex was competed away in the presence of excess unlabeled −55/−30 oligonucleotide (*lanes 4* and *5*), indicating that binding

FIG. 7. **Effect of internal deletions on basal activity and TPA-induced activity of the FGF-BP promoter.** The *left* histogram shows the impact of each mutation on basal activity of the construct (*center*). The control vector shown is the thymidine kinase minimal promoter in PXP1. The *right* histogram shows the transcriptional activity in the presence of TPA 10^{-7} M for each internal deletion of FGF-BP promoter. Each promoter construct was transiently transfected into ME-180 cells, and luciferase activity is expressed as fold induction of TPA-treated over untreated for each construct. Values represent the mean ± S.E. from at least three separate experiments, each done in triplicate wells. *Asterisk* indicates significant difference ($p < 0.05$) from the −118/+62 promoter construct.

Basal (%): 150 100 50 0

TPA Induction (Fold): 0 2 4 6 8 10 12 14 16 18 20

Ets Sp1 Ets AP1 C/EBPβ

Constructs: −118/+62, ΔEts, ΔAP1, ΔEts/ΔAP1, ΔC/EBPβ, Δ57/47, m-58, ΔC/EBPβ/m-58, −31/+62, control vector

was specific. Competition by the C/EBPβ site from the p21$^{WAF1/CIP1}$ gene promoter (30) for the specific complex was effective only at high molar excess (data not shown) indicating that the FGF-BP −55/−30 element may bind a different C/EBPβ-related factor.

To investigate further the transcriptional activation of the FGF-BP promoter by AP-1 and the involvement of the variant E box repressor element, gel shift experiments were carried out using the labeled promoter sequence spanning the juxtaposed AP-1/repressor element as a probe (−70/−51, Fig. 8*A*). In the presence of ME-180 nuclear extracts, the −70/−51 element bound an upper complex (Fig. 8*D, arrow*) and a lower doublet (*bracket*). The binding of all three complexes was highly induced by TPA (Fig. 8*D, lane 3*) and was effectively competed by molar excess of the unlabeled −70/−51 oligonucleotide (*lanes 4 and 5*). To understand better the specific composition of these complexes, point mutations were introduced in either the AP-1 site (mut AP-1) or the repressor site (mut −58) and tested for their abilities to compete for binding. Competition with the mutant AP-1 site resulted in a decrease of only the bottom doublet and no competition for the upper band (*lanes 6–8*), suggesting that the upper band corresponds to factors bound specifically to the AP-1 site. Conversely, when competition was carried out with the repressor mutant (mut −58), binding of the doublet on the probe remains intact, whereas binding to the AP-1 site is reduced (*lanes 9–11*), indicating that the lower two bands represent distinct protein binding to the repressor element. Furthermore, when competition was carried out with an AP-1 consensus, which contains an AP-1 site flanked by sequences which are not homologous to the FGF-BP promoter, competition for only the upper AP-1 complex was observed (*lanes 12* and *13*). These results show that the AP-1 site and the repressor site of the FGF-BP promoter bind distinct and specific transcription factor complexes that are induced in the presence of TPA. Taken together, our data show that sequences between −77 and −33 of the FGF-BP promoter form a novel TPA regulatory cassette consisting of interacting positive and negative control elements.

DISCUSSION

In this report we demonstrate that TPA induction of FGF-BP mRNA levels is primarily through stimulation of gene transcription. This is in contrast to the retinoid repression of FGF-BP gene expression which we have previously shown is

mediated through post-transcriptional and transcriptional mechanisms (7). In fact, at least at early time points after retinoid administration, the post- transcriptional mechanism which is dependent on new protein synthesis predominates since the half-life of the FGF-BP mRNA is greater than 16 h (7). Our studies show that the TPA induction of FGF-BP mRNA is rapid, requiring no new protein synthesis and involves direct activation by transcription factors whose site of action is clustered in the first 118 base pairs upstream of the transcription start site. Within this region the majority of the TPA stimulation of the FGF-BP promoter can be explained by the additive effects of two sites positioned between −76 to −58 and from −47 to −33.

The −76 to −58 site harbors a perfect consensus to the AP-1 transcription factor binding site NTGAGTCA (31). The AP-1 transcription factor complex comprises the c-*fos* and c-*jun* proto-oncogenes which are known to be activated as a result of TPA stimulation of PKC-dependent pathways (32). However, deletion of the AP-1 site alone in the FGF-BP promoter caused only a slight reduction in TPA effects on the FGF-BP promoter. This result is consistent with the emerging picture that AP-1 acts synergistically with other transcription factors, such as the Ets family of transcription factors, to mediate gene expression in response to TPA and other stimuli (28, 29). In the FGF-BP promoter deletion of sequences 5′ to the AP-1 consensus significantly decreases the TPA stimulation in comparison with deletion of the AP-1 site alone. These 5′ sequences contain the core GGA found in the center of the Ets family DNA consensus recognition site (29). Considering the body of evidence that suggests that Ets/AP-1 cooperate for full transcriptional activation, it seems likely that this may be the function of the −76/−58 element. For instance similar cooperation between Ets and AP-1 occurs through a juxtaposed Ets/AP-1-binding site in the polyoma virus enhancer (18) and has subsequently been implicated in the regulation of genes involved in invasion and metastasis, including collagenase and urokinase plasminogen activator (19, 33–37). Although we found that the collagenase Ets element or the polyoma virus Ets element did not effectively compete for binding to the FGF-BP Ets element, this may reflect the binding of another Ets family member to the FGF-BP promoter whose recognition site could be determined by sequences flanking the GGA core (27).

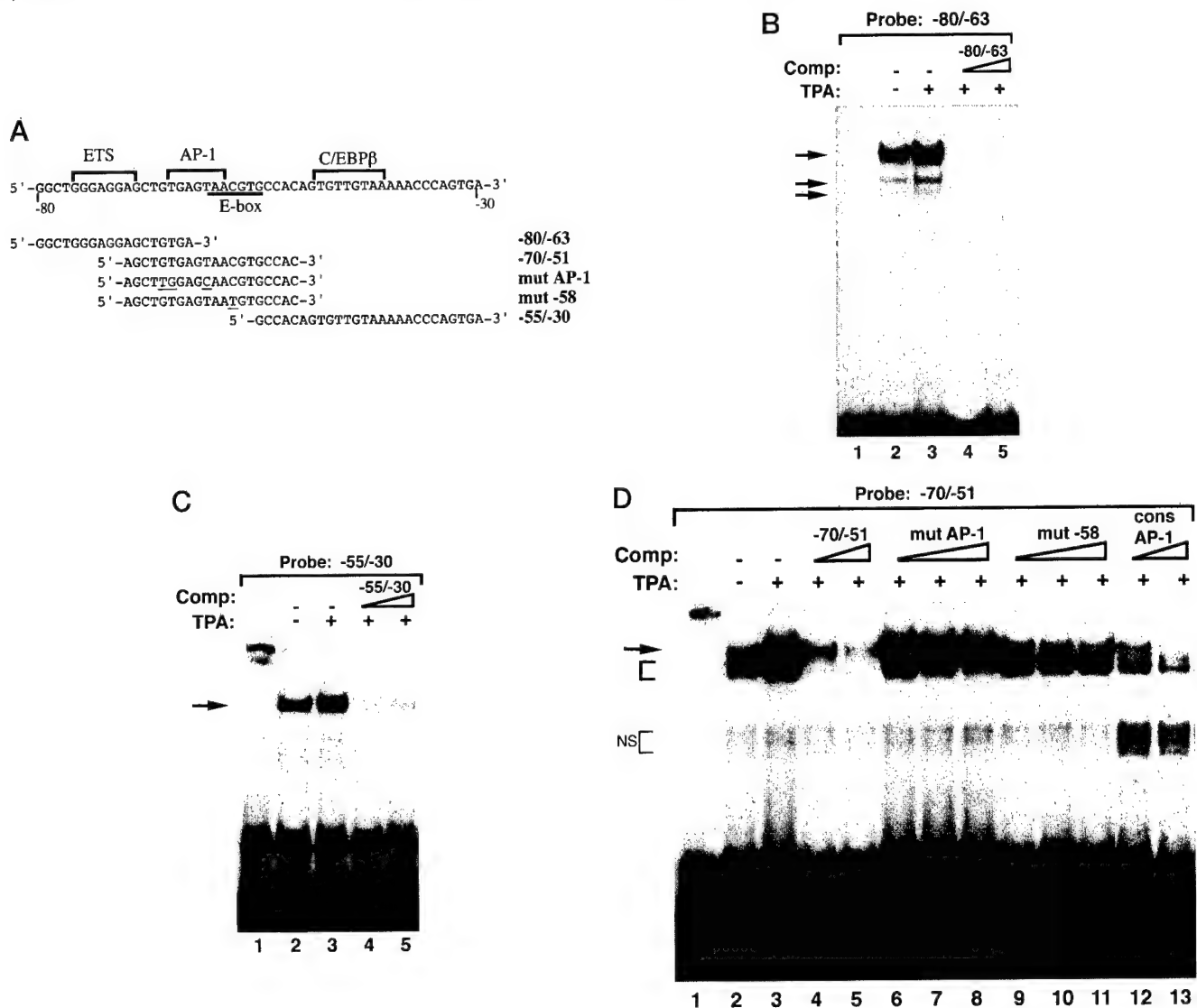Deletion of the −47 to −33 FGF-BP promoter region also

FIG. 8. **Transcription factor binding to FGF-BP promoter elements.** *A*, double-stranded oligonucleotide sequences used for gel shift analysis. Point mutations are *underlined*. *B*, gel shift assay with ^{32}P-labeled FGF-BP promoter sequence from −80 to −63 containing the putative Ets site either alone (*lane 1*) or in the presence of untreated (*lane 2*) or TPA-treated (*lanes 3–5*) ME-180 nuclear extracts. Binding reactions were incubated in the presence of 20-fold (*lane 4*) or 50-fold (*lane 5*) molar excess of the unlabeled −80/−63 oligonucleotide. *C*, gel shift assay with labeled promoter sequence from −55 to −30 containing the putative C/EBPβ-binding site either alone (*lane 1*) or in the presence of untreated (*lane 2*) or TPA-treated (*lanes 3–5*) ME-180 nuclear extracts. Competition for binding was carried out with 20-fold (*lane 4*) and 50-fold (*lane 5*) molar excess of unlabeled −55/−30 oligonucleotide. *D*, gel shift assay with labeled promoter sequence from −70 to −51 containing the AP-1 and repressor sites either alone (*lane 1*) or in the presence of untreated (*lane 2*) or TPA-treated (*lanes 3–13*) ME-180 nuclear extracts. Competition in the presence of 10-fold (*lanes 6 and 9*), 20-fold (*lanes 4, 7, 10,* and *12*), or 50-fold (*lanes 5, 8, 11,* and *13*) molar excess of unlabeled oligonucleotides as indicated. Specific complexes are indicated by an *arrow* on the *left* of each panel and nonspecific complexes are labeled *NS*.

substantially reduces the TPA effects on the FGF-BP promoter. Sequence analysis revealed that a site homologous to the C/EBPβ-binding site is centered in this region of the promoter. The factors binding to FGF-BP C/EBPβ element, however, are not effectively competed by the C/EBPβ site from the p21$^{WAF1/CIP1}$ gene promoter, suggesting that transcription of FGF-BP may be mediated by a different C/EBPβ-related factor. The published consensus for C/EBPβ is T(T/G)NNGNAA(T/G) (38) which is identical in eight positions (underlined) to the site between −48 to −41 differing only in the most 3′-nucleotide of the consensus. In addition, the involvement of C/EBPβ in TPA-mediated responses has been shown previously. For instance, induction by phorbol esters has been shown to cause increased C/EBPβ synthesis, phosphorylation, and DNA binding to promoters of a number of genes including MDR1 and collagenase 1 (21–23, 39). Thus, C/EBPβ or a family member is involved in the activity of the −47 to −33 element. Like other leucine

zipper family members, C/EBPβ acts cooperatively with other transcription factors to modulate the level of gene expression in response to extracellular stimuli. For example, C/EBPβ has been shown to associate with Fos/Jun *in vitro* (40) and can cooperate *in vivo* to induce expression of the *TSG-6* gene in response to interleukin-1 and tumor necrosis factor-α which is mediated through distinct AP-1 and C/EBPβ-binding sites in the *TSG-6* promoter (41). Similarly, our data show that the C/EBPβ consensus element is a major mediator of TPA-induced gene expression of FGF-BP. However, because removal of the C/EBPβ site alone does not completely abolish TPA induction, this suggests that like other TPA-induced genes, the C/EBPβ site acts in cooperation with other promoter elements.

A novel aspect of TPA regulation of the FGF-BP promoter is the role of the region −57 to −47 between the AP-1 site and the C/EBPβ site. Deletion of this region substantially increases the TPA response, implying that this region normally represses the

extent of the response to TPA. A point mutation in this region also abrogates repression thus making it unlikely that the effect of the deletion is simply to bring the AP-1 and C/EBPβ sites in closer proximity leading to their increased responsiveness to TPA. In fact, the relief of repression obtained with the -58 point mutant is observed in the presence of the C/EBPβ deletion suggesting that the repression impacts on the AP-1 element rather than the C/EBPβ site. An alternate possibility is that the factor bound to the -57 to -47 site interacts with the general transcription machinery in a manner similar to the NC2 repressor (42). However, this seems less likely because we observe no increase in basal activity of the promoter after deletion or mutation of the repressor site in comparison to the -118 construct (Fig. 7). The -57 to -47 deletion destroys an AACGTG (-60 to -55) which is a variant of the CACGTG E box element recognized by a number of helix-loop-helix factors (24). The -58 mutant changes the AACGTG to AATGTG and would perturb the 5' part of the dimer recognition sequence (24). However, the wild type sequence alone does not predict which member of the helix-loop-helix family would interact with this site. Interestingly, binding to an AACGTG recognition element has been described *in vitro* to a homodimer of the aryl hydrocarbon receptor nuclear translocator helix-loop-helix factor (43), and aryl hydrocarbon receptor nuclear translocator-deficient embryonic stem cells have a defective angiogenesis process (44). However, it is unclear whether aryl hydrocarbon receptor nuclear translocator homodimers interact with promoters *in vivo*. Alternatively, other helix-loop-helix factors are known to function as transcriptional repressors, such as the Mad family of proteins that bind related E box sequences during TPA-induced macrophage differentiation (45, 46) and recruit the mSin3-histone deacetylase corepressor complex, leading to a more closed chromatin structure and transcriptional repression (47).

Through gel retardation analysis, we show distinct factor binding to the AP-1 site and to the E box repressor site. Interestingly, factor binding to both of these sites is increased upon stimulation with TPA. TPA-induced transcription factor binding to E box elements has been described for a number of different promoters including c-*fos* (48–50). The observation that TPA induces factors which both stimulate and limit induction of FGF-BP suggests a mechanism by which transcription of the FGF-BP gene could be tightly regulated and may reflect a level of tissue-specific expression of this gene.

Overall, our data suggest that the TPA induction of the FGF-BP promoter is induced through both Ets/AP-1 site and a C/EBPβ site and that the extent of induction is moderated by factors that bind to an E box repressor element which lies adjacent to the AP-1 site. It is known that TPA also induces the expression of genes involved in proteolytic degradation of the extracellular matrix such as stromelysin, collagenase, and urokinase plasminogen activator (33, 51, 52). Interestingly, these promoters are regulated by similar transcription factors as those which we show are involved in FGF-BP promoter induction, *e.g.* Ets/AP-1 and C/EBPβ. Thus, our data would support the argument that a specific subset of transcription factors may be induced (or derepressed) to specifically stimulate a panel of genes involved in invasion, angiogenesis, and metastasis during skin tumor development.

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Induction of the Angiogenic Modulator Fibroblast Growth Factor-binding Protein by Epidermal Growth Factor Is Mediated through Both MEK/ERK and p38 Signal Transduction Pathways*

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Fibroblast growth factor-binding protein (FGF-BP) is a secreted protein that binds and activates fibroblast growth factors (FGF-1 and FGF-2) and induces angiogenesis in some human cancers. FGF-BP is expressed at high levels in squamous cell carcinoma (SCC) cell lines and tumor samples and has been shown to be rate-limiting for the growth of SCC tumors *in vivo*. In this study, we examine the regulation of FGF-BP by epidermal growth factor (EGF) and the signal transduction mechanisms that mediate this effect. We found that EGF treatment of the ME-180 SCC cell line caused a rapid induction of FGF-BP gene expression. This induction was mediated transcriptionally through the AP-1 (c-Fos/JunD) and CCAAT/enhancer-binding protein elements as well as through an E-box repressor site in the proximal regulatory region of the FGF-BP promoter. Pharmacological inhibition of protein kinase C and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 (MEK1/2) completely blocked EGF induction of FGF-BP mRNA, whereas inhibition of phosphatidylinositol 3-kinase had no effect. Additionally, both EGF- and anisomycin-induced FGF-BP mRNA was abrogated by inhibition of p38 mitogen-activated protein kinase, demonstrating a role for p38 in the regulation of FGF-BP. Co-transfection of the FGF-BP promoter with dominant negative forms of MEK2, extracellular signal-regulated kinase 2, and p38 significantly decreased the level of EGF induction, whereas expression of a dominant negative c-Jun N-terminal kinase mutant or expression of c-Jun N-terminal kinase inhibitory protein had no effect. Similarly, activation of the p38 pathway by overexpression of wild-type p38 or MKK6 enhanced FGF-BP transcription. These results demonstrate that EGF induction of FGF-BP occurs selectively through dual activation of the stress-activated p38 and the MEK/extracellular signal-regulated kinase mitogen-activated protein kinase pathways, which ultimately leads to activation of the promoter through AP-1 and CCAAT/enhancer-binding protein sites.

A pivotal process in a healing wound as well as in a growing tumor is the development of new blood vessels, or angiogenesis. Some of the most potent angiogenic stimulators are the fibroblast growth factors, including the classical angiogenic activators of this family, FGF-1[1] and FGF-2 (1). High concentrations of biologically active FGF-1 and FGF-2 are found in extracts of normal human tissues that are not necessarily undergoing active new blood vessel growth (1). This is due to the storage of FGF-1 and FGF-2 in the extracellular matrix, where they are found tightly bound to membrane-attached heparan sulfate proteoglycans (2), which quenches their biological activity. One mechanism by which FGF-1 and FGF-2 are released from the extracellular matrix is through the secretion of the carrier protein FGF-BP that binds to FGF-1 and FGF-2 in a noncovalent and reversible manner (3). FGF-BP is actively secreted from cells and, once bound, prevents degradation of FGF-1 and FGF-2 (3, 4). The importance of FGF-BP secretion in promoting tumor growth was shown in studies using the nontumorigenic adrenal carcinoma cell line SW-13, which has high expression of FGF-2 but is negative for FGF-BP. Stable overexpression of FGF-BP in SW-13 cells led to a dramatic increase in FGF-2-dependent colony formation and formation of highly vascularized tumors in nude mice (4). Additional studies were carried out to characterize the biological role of FGF-BP in highly tumorigenic cell lines such as ME-180 (human cervical squamous cell carcinoma) and LS174T (colon adenocarcinoma), which express high levels of endogenous FGF-BP (4, 5). Reduction of FGF-BP mRNA levels in these cell lines using ribozyme targeting significantly inhibited tumor development and angiogenesis (6). These studies demonstrated that FGF-BP serves as a rate-limiting angiogenic modulator for some tumor types (6, 7).

FGF-BP is highly expressed in squamous cell carcinoma (SCC) cell lines from lung, bladder, skin, and cervix and is positive in primary SCC tumor samples (4). Furthermore, its expression has been shown to be up-regulated during mouse embryonic development of the skin, lung, and intestine and is low in most adult tissues (8). A potential role for FGF-BP during skin carcinogenesis was described in studies showing dramatic FGF-BP up-regulation in human[2] and mouse skin

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[1] The abbreviations used are: FGF, fibroblast growth factor; FGF-BP, fibroblast growth factor-binding protein; SCC, squamous cell carcinoma; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; C/EBP, CCAAT/enhancer-binding protein; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKC, protein kinase C; PI, phosphatidylinositol; JNK, c-Jun N-terminal kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; IMEM, improved minimum essential medium; JIP, JNK-inhibitory protein; MAP, mitogen-activated protein.

[2] A. Aigner and A. Wellstein, manuscript in preparation.

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treated topically with DMBA and TPA (8). These observations suggested several mechanisms that might be involved in the direct regulation of the FGF-BP gene. First, DMBA treatment has been shown to cause a specific point mutation in the *ras* oncogene (9), suggesting that the Ras signal transduction pathway might regulate FGF-BP expression. This is also indicated by the observation that FGF-BP is induced in *ras*-transformed keratinocytes (8). Second, the role of TPA as a direct regulator of FGF-BP gene expression was confirmed upon TPA treatment of several SCC cell lines, including ME-180, which caused rapid transcriptional induction of the FGF-BP gene (10). We further identified that Sp1, AP-1, and C/EBP sites within the proximal FGF-BP promoter are all required for TPA regulation of FGF-BP (10).

SCC cell lines and tumors, including the ME-180 cell line, typically express high levels of EGF receptors (EGFRs) (11, 12), and overexpression of EGFR in SCC has been shown to confer greater tumorigenicity (13), which led us to investigate the possible role and mechanisms of EGF regulation of FGF-BP. EGF signaling occurs predominantly through binding to its receptor EGFR (HER1) and its dimerization partner ErbB2 (HER2/Neu). Autophosphorylation of activated EGF receptors stimulates a number of signal transduction pathways, including the classical Ras/Raf/MAP kinase kinase (MEK)/MAP kinase (ERK) pathway, which is known to phosphorylate and activate AP-1 (14). MEK/ERK activation occurs either through phosphorylated EGFR recruitment of the Shc-Grb2-SOS complex and subsequent Ras activation or through recruitment of phospholipase Cγ and subsequent PKC activation. PKC can in turn modulate Raf through both Ras-dependent and -independent mechanisms (15, 16). Other signaling pathways induced by EGF include the stress-activated protein kinases such as JNK and p38 (17), the PI 3-kinase pathway (18), and the Janus kinase/signal transducers and activators of transcription pathway (19).

Here we show a possible link between EGF signaling and angiogenic activation through the regulation of the FGF-BP gene in SCC. We found that EGF treatment induces rapid up-regulation of FGF-BP transcription occurring through the AP-1 and C/EBP sites in the FGF-BP promoter. Furthermore, inhibition of either the MEK/ERK pathway or the p38 pathway abrogates induction by EGF, implicating dual activation of these MAP kinases as an important step in FGF-BP regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The ME-180 cervical squamous cell carcinoma cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in improved minimum essential medium (IMEM) (Biofluids Inc., Rockville, MD) with 10% fetal bovine serum (Life Technologies, Inc.). Actinomycin D, calphostin C, and wortmannin were purchased from Sigma. Anisomycin and tyrphostin AG1478 were from Alexis Corp. SB203580, SB202190, and SB202474 were from Calbiochem, and U0126 was purchased from RBI (Natick, MA). All compounds were dissolved in Me$_2$SO.

Northern Analysis—ME-180 cells were grown to 80% confluence in 10-cm dishes, washed twice in serum-free IMEM, and incubated for 16 h in serum-free IMEM prior to treatment. Cells were pretreated for 1 h with the indicated drug or with vehicle alone (Me$_2$SO; final concentration 0.1%). EGF or anisomycin treatment was for 6 h unless otherwise indicated. Total RNA was isolated with RNA STAT-60™ (Tel-Test Inc.), and Northern analysis was carried out as described previously (10). Hybridization probes were prepared by random-primed DNA labeling (Amersham Pharmacia Biotech) of purified insert fragments from human FGF-BP (4) and human GAPDH (CLONTECH). Quantitation of mRNA levels was performed using a PhosphorImager (Molecular Dynamics, Inc.).

Plasmids—Human FGF-BP promoter fragments were cloned into the pXP1 promoterless luciferase reporter vector and have been described previously (10). The mutant AP-1 FGF-BP promoter construct was generated by PCR as described previously (10), introducing point mutations that convert the AP-1 site from GTGAGTAA (−66 to −59) to

TGGAGCAA. The MEK2 (K101A) dominant negative was provided by Dr. J. Holt (Vanderbilt University) (20). The dominant negative of ERK2 (K52R) and the empty vector pCEP4 were provided by Dr. M. Cobb (University of Texas Southwestern) (21). The expression plasmids containing dominant negative JNK1 (APF), dominant negative p38α (AGF), wild-type JNK (pCDNA3-Flag-JNK1), wild-type p38 (pCDNA3-Flag-p38), JIP (pCDNA3-Flag-JIP1), and constitutively active MKK6 (pCDNA3-Flag-MKK6(Glu)) were provided by Dr. R. Davis (University of Massachusetts) (22–25). All effects of dominant negatives were compared with their empty vector control or with the empty vector pCDNA3 (Invitrogen).

Transient Transfections and Reporter Gene Assays—24 h before transfection, ME-180 cells were plated in six-well plates at a density of 750,000 cells/well. pRL-CMV *Renilla* luciferase reporter vector (Promega) was included as a control for transfection efficiency. For each transfection, 1.0 µg of FGF-BP promoter-luciferase construct, 0.1 ng of pRL-CMV (transfection efficiency control), and 8 µl of LipofectAMINE (Life Technologies, Inc.) were combined and added to cells for 3 h in serum-free conditions as described previously (10). For co-transfections, 1.0 µg of −118/+62Luc FGF-BP promoter construct, 500 ng of expression vector, 0.1 ng of pRL-CMV, and 8 µl of LipofectAMINE were added to cells. Transfected cells were treated for 18 h with EGF (5 ng/ml) in serum-free IMEM before cell lysis in 150 µl of passive lysis buffer (Promega). 20 µl of extract was assayed for both firefly and *Renilla* luciferase activity using the Dual-Luciferase™ reporter assay system (Promega). Due to a small background induction (1.5–2 fold) of the pRL-CMV plasmid by EGF, all luciferase values were normalized for protein content. There were no significant differences, however, in the transfection efficiencies between plasmid constructs in untreated controls as determined by *Renilla* luciferase assay. Protein content of cell extracts was determined by Bradford assay (Bio-Rad).

Gel Shift Assays—ME-180 cells were grown to 80% confluency on 150-mm dishes, serum-starved in IMEM for 16 h, and treated with or without 5 ng/ml EGF for 1 h. Nuclear extracts were prepared as described previously (10). Binding reactions with −70/−51 and −80/−63 probes were carried out as described previously (10) with 5 or 1 µg, respectively, of ME-180 nuclear extracts, binding buffer (20 mM Tris, pH 7.5, 60 mM KCl, 5% glycerol, 0.5 mM dithiothreitol, 2.0 mM EDTA), and 250 ng of poly(dI-dC). Binding reaction with −55/−30 probe was carried out with 5 µg of ME-180 nuclear extracts and 500 ng of poly(dI-dC). Supershift antibodies (2 µg) were added to the binding reaction for 10 min on ice before adding 20 fmol of labeled probe. Reactions were carried out 45 min on ice and analyzed by 6% polyacrylamide gel electrophoresis. Supershift antibodies purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were the following: Fos-specific antibodies c-Fos (K-25), c-Fos (4), Fos B (102), Fra-1 (R-20), and Fra-2 (Q-20); Jun-specific antibodies c-Jun/AP-1 (D), c-Jun/AP-1 (N), JunB (N-17), and JunD (329); Sp1-specific antibody Sp1 (PEP 2); and C/EBP-specific antibodies C/EBP α (14AA), C/EBPβ (C-19), C/EBPβ (Δ198), C/EBPγ (C-20), C/EBPδ (M-17), and C/EBPε (C-22). For oligonucleotide competition, a 50-fold molar excess of unlabeled double-stranded competitor was added to the binding reaction and incubated for 10 min on ice before the addition of the labeled probe. Sequence of the C/EBP consensus fragment was 5′-TGCAGATTGCGCAATCTGCA-3′ (Santa Cruz Biotechnology).

Immunocomplex Akt Kinase Assay—ME-180 cells were grown in 150-mm dishes and pretreated as described above for Northern analysis. Cells were treated with EGF (5 ng/ml) for 5 min. Cell lysates were prepared by scraping the cells into immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 1 µM okadaic acid, 50 mM sodium fluoride, 2 µg/ml aprotinin, 1 µg/ml pepstatin A) and incubating for 15 min at 4 °C in a rotating rack. The lysates were cleared by centrifugation, and protein content was measured with the Bio-Rad protein assay kit. Lysates were precleared with protein G-Sepharose and incubated for 4 h at 4 °C with 3 µg of sheep anti-Akt1 antibody (Upstate Biotechnology, Inc., Lake Placid, NY). Immunocomplexes were captured with protein G-Sepharose at 4 °C for 1 h. The beads were then washed with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 1 mM dithiothreitol. The Akt kinase assay was carried out as described previously (26), using the K9 peptide as a substrate.

RESULTS

EGF Treatment Increases FGF-BP mRNA in SCC Cells—We have shown previously that phorbol ester (TPA) treatment of the human cervical SCC cell line ME-180 results in a time- and dose-dependent increase of FGF-BP mRNA, which is mediated
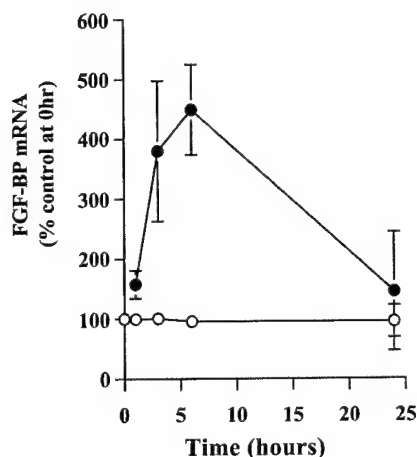
FIG. 1. **Induction of FGF-BP mRNA by EGF in ME-180 cells.** Shown is Northern analysis of FGF-BP mRNA levels in ME-180 cells that were either untreated or treated with 5 ng/ml EGF for the indicated amounts of time. Northern blot signal intensities of FGF-BP mRNA were quantitated by PhosphorImager and normalized to GAPDH. *Open circles* represent control (untreated) levels, and closed circles represent EGF treatment. Values represent the mean and S.D. of at least two separate experiments.

via the PKC signal transduction pathway (10). Similarly, treatment of ME-180 cells with EGF resulted in a rapid increase in the steady-state levels of FGF-BP mRNA with no effect on GAPDH mRNA levels (Fig. 1). FGF-BP mRNA induction was detectable after 1 h of treatment and was maximal after 6 h with an average increase of 4.5-fold. The rapid and transient nature of EGF induction of FGF-BP mRNA is identical to that seen after TPA treatment (10), suggesting that these agents might induce FGF-BP through a similar transcriptional mechanism.

To determine whether FGF-BP is up-regulated by EGF at the transcriptional level, we tested the effect of the transcription inhibitor actinomycin D on the EGF induction of FGF-BP mRNA. Pretreatment with actinomycin D completely blocked the induction of FGF-BP by EGF (see Fig. 4). Combined treatment with EGF and cycloheximide had no effect on induction of FGF-BP mRNA (data not shown), indicating that *de novo* protein synthesis is not required for the EGF response. Furthermore, transient transfection of the full-length FGF-BP promoter from -1060 to $+62$ into ME-180 cells resulted in a 4.5-fold increase in luciferase activity upon EGF treatment (Fig. 2). These data demonstrate that EGF, like TPA, can directly increase the rate of FGF-BP gene transcription.

Identification of EGF Response Elements within the FGF-BP Promoter—Functional analysis of the FGF-BP promoter has shown that TPA-induced transcription involves a combination of both positive and negative regulatory elements located within the first 118 base pairs of the proximal promoter (10). Full TPA induction was mediated by a C/EBP consensus site between -48 and -40, as well as through a juxtaposed Sp1/AP-1 element between -76 and -59. The Sp1(b) site between -76 and -68 was described previously as an Ets element based on its homology to other Ets consensus binding sites (10). However, we have now determined through supershift analysis that this site is bound by the Sp1 transcription factor (Fig. 3D). To investigate whether these same regulatory elements play a role in EGF induction of the FGF-BP promoter, we transiently transfected a series of mutated promoter constructs into ME-180 cells and tested their ability to be induced after treatment with EGF. Deletion of promoter sequences from -1060 to -118 had no effect on the level of promoter activity and resulted in a similar 5-fold EGF induction (Fig. 2), demonstrating that the

EGF regulatory region of the promoter is located within the first 118 base pairs of the promoter. Although the upstream Sp1 site (Sp1(a)) drives a significant portion of basal promoter activity (10), deletion of this site had no effect on EGF induction (data not shown). Deletion of the Sp1(b) site within the context of the $-118/+62$ promoter fragment also had no effect on EGF induction (Fig. 2). Basal activity of the promoter, however, did drop significantly in the absence of the Sp1(b) element (Fig. 2 and Ref. 10). In contrast, mutation of the AP-1 site resulted in a significant 40% decrease in EGF induction as compared with the wild-type $-118/+62$ promoter. Similarly, complete deletion of the juxtaposed Sp1(b)/AP-1 site resulted in a 46% decrease in EGF response. Mutation of the AP-1 site had no effect on basal promoter activity, demonstrating that changes in EGF responsiveness can occur independently of changes in the basal rate of transcription. These results show that unlike TPA induction, which requires the entire Sp1(b)/AP-1 element, EGF induction is driven mainly through the AP-1 site in the promoter, reflecting possible differences in the mechanisms by which each of these agents regulate the transcription of FGF-BP.

To test the contribution of the C/EBP element to EGF induction, we introduced an internal deletion of this site within the context of the $-118/+62$ promoter. Removal of the C/EBP site significantly reduced the EGF effect by 46% but had no influence on basal promoter activity (Fig. 2). Together, these data demonstrate the requirement for an intact C/EBP site and AP-1 site in the EGF induction of the FGF-BP promoter.

In addition to these positive regulatory elements, we recently showed that TPA regulation of the FGF-BP promoter involves a negative regulatory element, lying just downstream of the AP-1 site, which shows similarity to an E-box factor binding site (10). We wanted to determine whether this E-box repressor site also played a role in the regulation by EGF. Point mutation of this site at position -58, or deletion of this site from -57 to -47 resulted in a dramatic increase in EGF induction of the promoter from about 5-fold in the wild-type promoter to about 8–10-fold in the repressor mutants (Fig. 2), indicating loss of repressor activity. Therefore, the repressor element between -58 and -47 also plays a regulatory role in EGF induction by limiting the transcriptional response to growth factor stimulation.

AP-1, C/EBP, and Sp1 Binding to the FGF-BP Promoter—Because the AP-1 site in the FGF-BP promoter appears to be important during TPA and growth factor regulation of FGF-BP, we determined which members of the Fos and Jun family might be binding to the FGF-BP AP-1 site. To identify transcription factor binding to the AP-1 site, we carried out gel shift analysis using labeled promoter sequence fragment from -70 to -51 (Fig. 3A), which was incubated in the presence of nuclear extracts from control or EGF-treated ME-180 cells. Protein binding in the uppermost complex, which has previously been shown to represent AP-1 (10), is highly induced by EGF treatment (Fig. 3B, *lanes 1* and *2*). To determine the composition of the AP-1 complex, we used Fos and Jun-specific antibodies for supershift analysis. As shown in Fig. 3B, the addition of a Fos antibody that recognizes all Fos family members (*lane 3*) or that specifically recognizes c-Fos (*lane 4*) resulted in a supershifted complex. Antibodies against FosB, Fra-1, or Fra-2 had no effect. Furthermore, incubation with a general Jun family antibody (Fig. 3B, *lane 8*) or with a JunD-specific antibody (*lane 11*) either blocked or supershifted the AP-1 complex, respectively. The c-Jun- or JunB-specific antibodies had no effect on AP-1 binding. These results demonstrate that c-Fos and JunD are the major components of AP-1 binding to the FGF-BP promoter.
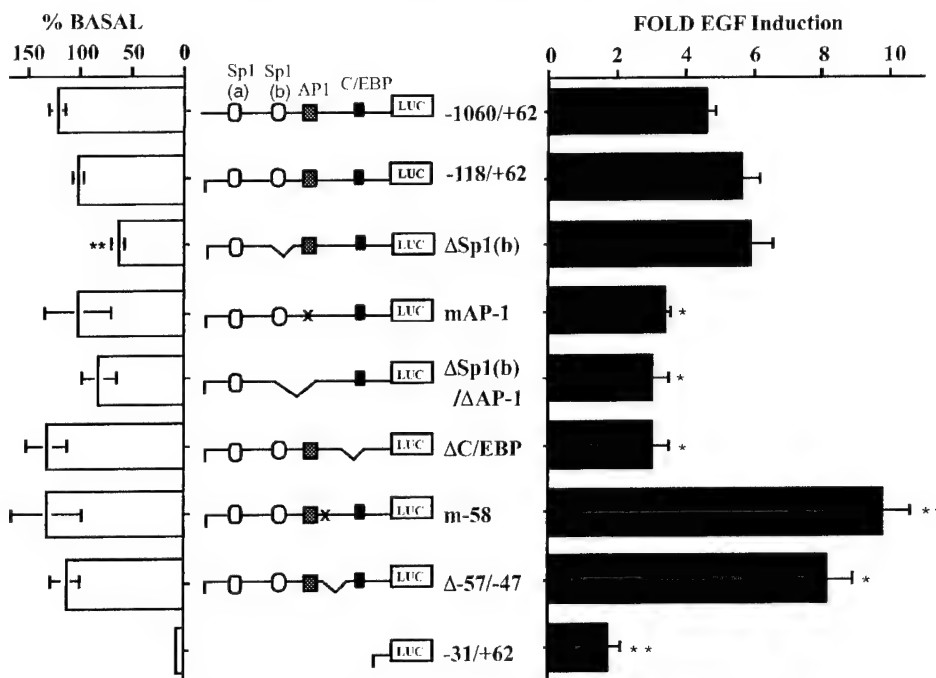
The binding of C/EBP to the FGF-BP promoter was investi-

FIG. 2. **Effect of FGF-BP promoter mutations on the transcriptional induction by EGF.** The *histogram* on the *left* shows the impact of each promoter deletion on the basal (uninduced) luciferase activity of each construct. The basal activity of the −118/+62 construct was set at 100%. The *right histogram* shows the transcriptional activity in the presence of EGF and is expressed as -fold induction of EGF-treated over untreated for each construct. ME-180 cells were transiently transfected with the indicated FGF-BP promoter luciferase constructs and were either untreated or treated with 5 ng/ml of EGF for 18 h. Promoter constructs are described under "Experimental Procedures" and in Ref. 10. Values represent the mean and S.E. from at least three separate experiments, each done in triplicate wells. Statistically significant differences relative to the −118/+62 promoter construct are indicated (*, $p < 0.05$; **, $p < 0.01$, t test).

gated using a promoter fragment spanning the C/EBP site from −55 to −30 (Fig. 3*A*). Gel shift analysis with this fragment in the presence of nuclear extracts from untreated or EGF-treated ME-180 cells showed no significant change in the binding of C/EBP (Fig. 3*C*, *lanes 1* and *2*). This finding is consistent with previously published observations that PKC activation phosphorylates and enhances C/EBP transcriptional activity with no effect on DNA-binding activity (27). The specificity of C/EBP binding in the upper complex of the gel shift (Fig. 3*C*, *arrow*) is demonstrated through competition with a molar excess of unlabeled −55/−30 fragment (*lane 9*) as well as with a C/EBP consensus element (*lane 10*). Incubation with an antibody against C/EBPβ that also cross-reacts with other members of the C/EBP family resulted in one prominently supershifted band (Fig. 3*C*, *lane 3*). In addition, antibodies specific for C/EBPβ (*lane 4*) and C/EBPδ (*lane 7*) also supershifted the complex, whereas antibodies for C/EBPα (*lane 5*), C/EBPγ (*lane 6*), and C/EBPε (*lane 8*) had little effect on C/EBP binding. Because the C/EBP site is required for full EGF induction of the FGF-BP promoter (Fig. 2), these results suggest that C/EBPβ and -δ are involved in mediating the EGF effect. The mechanism by which these factors are activated in response to EGF, however, remains to be determined.

As mentioned previously, the promoter element between −80 and −63 shows some homology to an Ets factor binding site. Closer analysis of this site also revealed homology to a variant Sp1 site, which was previously shown to respond to EGF treatment (28). In fact, supershift analysis of the −80 to −63 element showed specific binding of Sp1 (Fig. 3*D*). This element has been denoted Sp1(b) in order to distinguish it from the Sp1 site further upstream (Sp1(a)). Interestingly, the binding of Sp1 to the Sp1(b) site increased in the presence of nuclear extracts from EGF-treated cells (Fig. 3*D*, *lanes 1* and *2*) despite the fact the removal of the Sp1(b) site alone has no impact on the overall EGF induction of the FGF-BP promoter (Fig. 2).

Role of PKC and MEK1/2 Signal Transduction Pathways in FGF-BP Regulation—In order to differentiate between the possible signaling pathways involved in EGF induction of FGF-BP, we tested pharmacological inhibitors of signal transduction components for their effect on FGF-BP regulation. We found that treatment with the EGFR tyrosine kinase inhibitor tyrphostin AG1478 reduced EGF induction of FGF-BP to 30% (Fig. 4), which was not significantly different from the basal level of expression (without EGF or drug treatment) of approximately 25% (data not shown). As expected, EGFR tyrosine kinase activity is essential for the EGF effect on the FGF-BP gene. In addition, we have shown previously that TPA induction of FGF-BP transcription was mediated through a PKC-dependent pathway (10). To establish whether PKC activation was also required for the EGF effects on FGF-BP, we treated ME-180 cells with the specific PKC inhibitor calphostin C (29) and found that this completely blocked EGF induction of FGF-BP mRNA (Fig. 4). Therefore, PKC activation is central in mediating FGF-BP transcriptional activation upon either EGF or TPA stimulation.

Since the MEK/ERK pathway is known to be stimulated by EGF, we investigated the contribution of the MAP kinase kinases MEK1 or MEK2 to FGF-BP using pharmacological inhibitors of MEK1/2. Treatment with the drug U0126, which is a potent inhibitor of both MEK1 and MEK2, abrogated EGF induction of FGF-BP mRNA (Fig. 4). Consistent with the role of MEK in FGF-BP induction, treatment with a less potent MEK inhibitor PD98059 also blocked the EGF effect on FGF-BP, albeit at higher concentrations (data not shown). Therefore, activation of the MEK pathway is a necessary step in EGF regulation of FGF-BP.

EGF is also known to stimulate intracellular signaling via the PI 3-kinase pathway (18). In order to test the contribution of PI 3-kinase to FGF-BP regulation, we used the drug wortmannin to specifically inhibit PI 3-kinase activity. Pretreat-
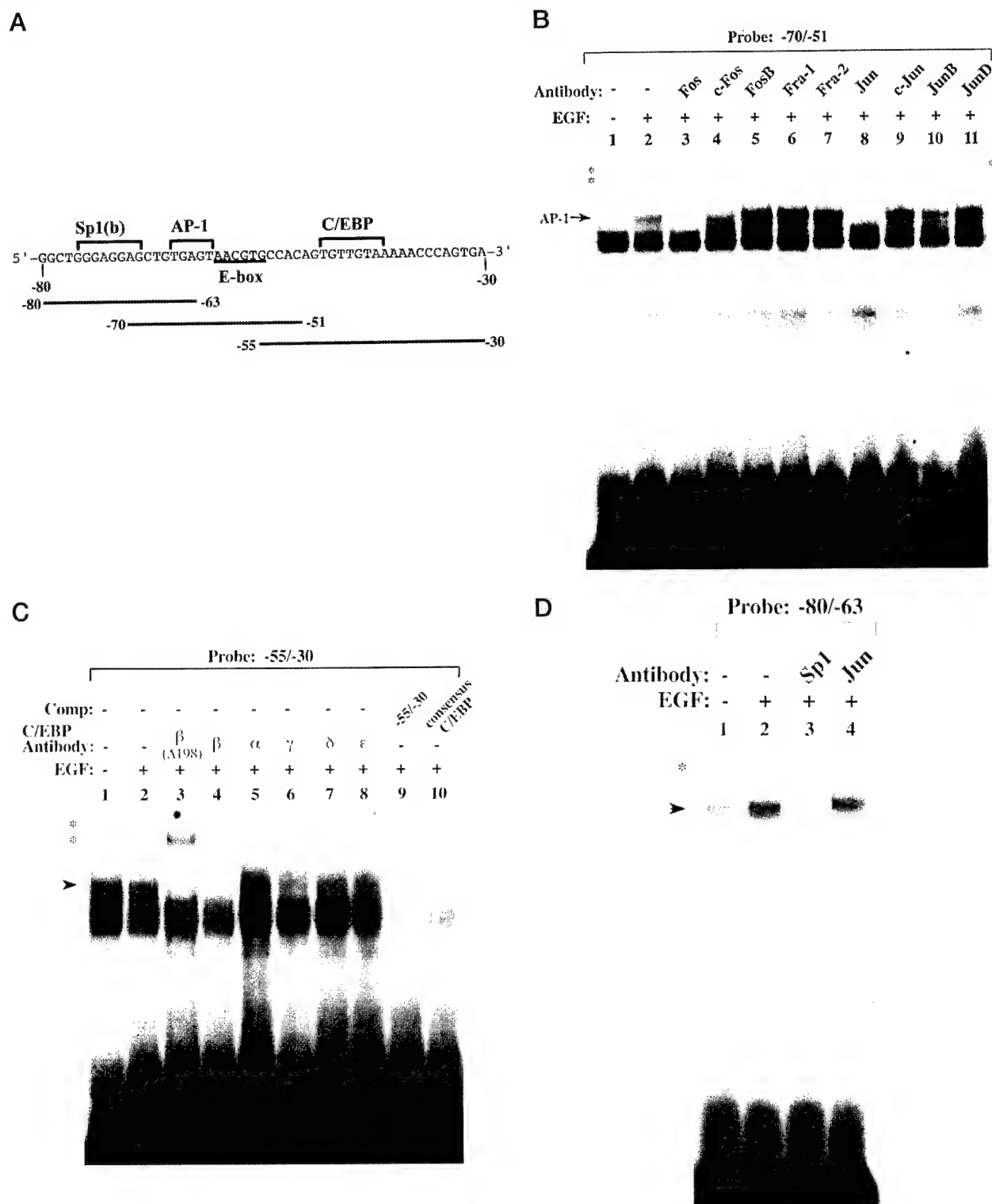
FIG. 3. **Characterization of transcription factor binding to FGF-BP promoter elements.** *A*, double-stranded oligonucleotide sequences of FGF-BP promoter elements used for gel shift analysis. Supershift analysis of transcription factor binding to the AP-1 site (*B*), C/EBP site (*C*), or Sp1(b) site (*D*) of the FGF-BP promoter. Gel shift assay with the labeled FGF-BP promoter sequences as indicated were incubated in the presence of nuclear extracts from untreated or EGF-treated ME-180 cells. Binding reactions were incubated in the presence of supershift antibodies as indicated in each figure. Specific binding of AP-1, C/EBP, and Sp1 are indicated by an *arrow* at the *left* of each *panel*. Supershift complexes are labeled by an *asterisk* on either side of the gel. Competition for C/EBP binding was carried out in the presence of a 50-fold molar excess of unlabeled oligonucleotides as indicated.

ment with wortmannin at two different concentrations had no effect on EGF induction or FGF-BP mRNA (Fig. 5*A*). Endogenous PI 3-kinase activity in ME-180 cells, as measured by immunoprecipitated Akt kinase activity, was induced approximately 2-fold by EGF and was effectively blocked by the same

concentrations of wortmannin and under the same experimental conditions used for the analysis of FGF-BP (Fig. 5*B*). Therefore, we conclude that activation of PI 3-kinase upon EGF treatment does not play an essential role in the regulation of FGF-BP expression. Furthermore, the lack of effect by wort-
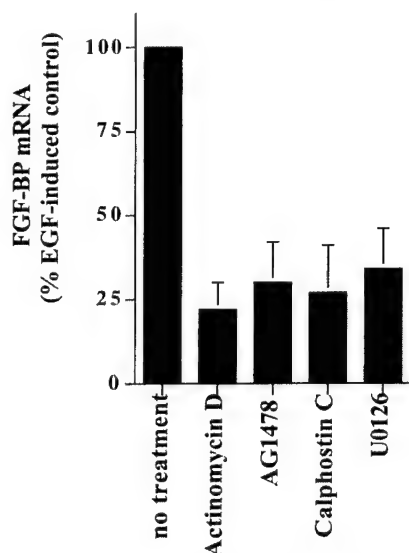
FIG. 4. **Effect of signal transduction inhibitors on EGF induction of FGF-BP mRNA.** FGF-BP mRNA levels from ME-180 cells treated with 5 ng/ml EGF for 6 h. Cells were pretreated for 1 h with vehicle alone, 5 μg/ml actinomycin D (transcription inhibitor), 100 μM tyrphostin AG1478 (EGFR tyrosine kinase inhibitor), 100 nM calphostin C (PKC inhibitor), or 10 μM U0126 (MEK1/2 inhibitor). Northern blot signal intensities of FGF-BP mRNA were quantitated, normalized to GAPDH, and expressed relative to mRNA levels after EGF treatment alone (without inhibitor), which was set to 100%. Basal FGF-BP level (without EGF or inhibitor) was approximately 25%. Values represent the mean and S.D. of at least two separate experiments.

mannin served as a negative control and demonstrates specificity of the inhibitions observed in Fig. 4.

Induction of FGF-BP through p38 Kinase—Stimulation of the JNK/p38 MAP kinase pathway has also been shown to regulate AP-1 activity in response to mitogens and stress (17). We therefore tested whether JNK or p38 activation could induce FGF-BP gene expression by treating with the antibiotic anisomycin. Anisomycin treatment at concentrations below 200 nM is known to be an effective stimulator of both JNK and p38 kinase activity (30). Treatment of ME-180 cells with anisomycin alone resulted in a significant and dose-dependent increase of FGF-BP mRNA levels up to 2.3-fold (Fig. 6A), suggesting that activation of the JNK/p38 pathway might be involved in the regulation of FGF-BP.

To further investigate the involvement of p38 kinase in FGF-BP regulation, we tested the contribution of p38 to FGF-BP induction using the p38-specific inhibitors SB203580 and SB202190, which have no inhibitory activity for JNK or ERK1/2 (31–33). Treatment with SB203580 or SB202190 significantly reduced EGF induction of FGF-BP mRNA by 50% to 75% in a dose-dependent manner (Fig. 6B). In contrast, there was no reduction after treatment with SB202474, a drug with a similar structure as SB203580 and SB202190 but with no inhibitory activity for p38. Additionally, p38 inhibition completely blocked anisomycin induction of FGF-BP (Fig. 6B, *inset*), demonstrating that both anisomycin and EGF induction of FGF-BP mRNA require p38 activation.

To investigate a possible additive or synergistic interaction between ERK and p38 pathways in EGF-induced FGF-BP expression, we treated cells simultaneously with the MEK inhibitor (U0126) and the p38 inhibitor (SB202190) and examined the effect on FGF-BP mRNA. As shown in Fig. 7, treatment with suboptimal doses of U0126 alone or with SB202190 alone resulted in approximately 20% decrease in EGF-induced FGF-BP. Simultaneous treatment with both inhibitors resulted in a 40% reduction of FGF-BP mRNA levels, indicating that the
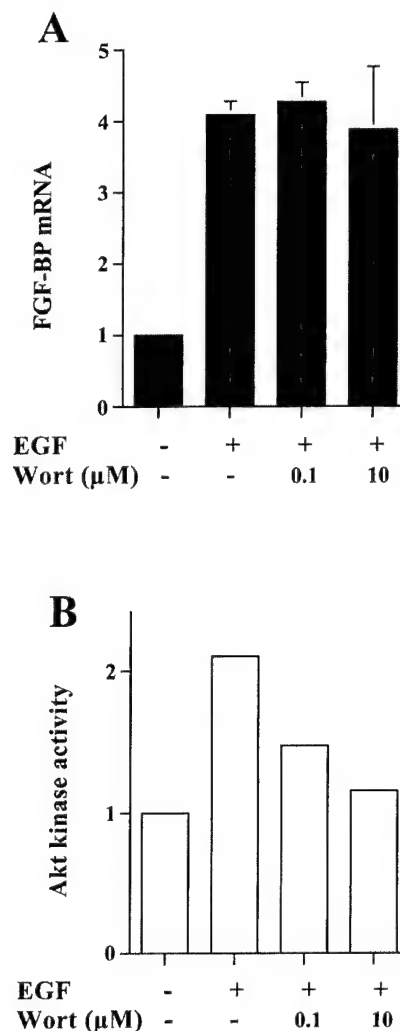

FIG. 5. **Effect of PI 3-kinase inhibition on EGF-induced FGF-BP mRNA.** *A,* FGF-BP mRNA levels after pretreatment with vehicle alone or with the indicated concentration of wortmannin (PI 3-kinase inhibitor) for 1 h, followed by 6 h of treatment with 5 ng/ml EGF. FGF-BP mRNA levels were analyzed by Northern blot, normalized for GAPDH, and expressed relative to untreated control. B, inhibition of PI 3-kinase activity by wortmannin. ME-180 cells were pretreated for 1 h with or without wortmannin, stimulated for 5 min with 5 ng/ml EGF, and assayed for Akt kinase activity (downstream target of PI 3-kinase) as described under "Experimental Procedures."

contribution of each of these pathways is additive and not synergistic.

Contribution of MEK/ERK and p38 Pathways to FGF-BP Promoter Activity—Inhibition of MEK and p38 with pharmacological agents demonstrated the importance of these kinases in mediating the induction of FGF-BP by EGF. To confirm the role of each of these pathways in the regulation of FGF-BP transcription, we expressed dominant-negative mutants that specifically suppress the enzymatic activity of their endogenous counterparts. Co-transfection of a dominant-negative form of MEK2 with the FGF-BP promoter luciferase construct (−118/+62) into ME-180 cells resulted in a significant decrease in the amount of EGF induction (Fig. 8A). In addition, inhibition of ERK, the MAP kinase target of MEK, by co-transfection of a kinase-deficient dominant negative ERK2 mutant, also resulted in a significant decrease in EGF induction (Fig. 8A). Inhibition of the MEK/ERK signaling pathway with these dominant negative mutants led to nearly a 50% decrease in EGF induction of the FGF-BP promoter. These results support the hypothesis that activation of MEK and ERK by EGF are nec-
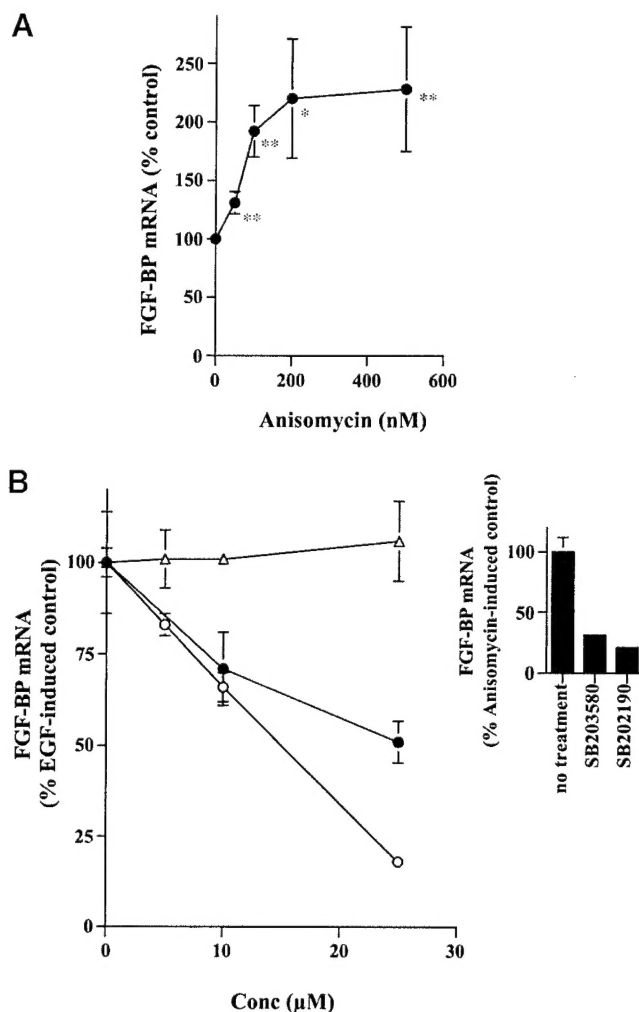
A

B

FIG. 6. **Involvement of p38 MAP kinase in the anisomycin and EGF induction of FGF-BP mRNA.** *A*, anisomycin induction of FGF-BP mRNA. ME-180 cells were treated for 6 h with the indicated concentrations of anisomycin. The mean and S.E. of at least three separate experiments are given. Statistically significant differences relative to the control (untreated) group are indicated (*, $p < 0.05$; **, $p < 0.01$, t test). *B*, p38 inhibition blocks EGF and anisomycin (*inset*) induction of FGF-BP mRNA. ME-180 cells were pretreated for 1 h with the indicated concentration of p38 inhibitors SB203580 (*closed circles*) and SB202190 (*open circles*) or with the control compound SB202474 (*open triangles*), followed by 6-h treatment with EGF (5 ng/ml) or anisomycin (200 nM). Northern blot signal intensities of FGF-BP mRNA were quantitated, normalized to GAPDH, and expressed relative to mRNA levels after EGF or anisomycin treatment alone (without inhibitor), which was set to 100%. Basal FGF-BP level (without EGF or inhibitor) was approximately 25% of EGF-treated. Values represent the mean and S.D. of at least two separate experiments.

essary for induction of the FGF-BP gene.

To test the contribution of p38 and JNK to EGF induction of FGF-BP, we co-transfected dominant negative mutants of JNK and p38 along with the FGF-BP promoter into ME-180 cells (Fig. 8A). Each dominant negative mutant contains an alanine and phenylalanine in place of the activating threonine or tyrosine phosphorylation sites (22, 23). Expression of the dominant-negative JNK mutant had no significant effect on EGF induction compared with the empty vector. On the other hand, expression of the dominant-negative p38 mutant consistently reduced EGF induction of FGF-BP transcription. Although inhibition of p38 reduced promoter activity by only 23%, this difference was statistically significant from empty vector-transfected cells. Additionally, we tested whether overexpression of wild-type JNK or p38 constructs could affect EGF in-
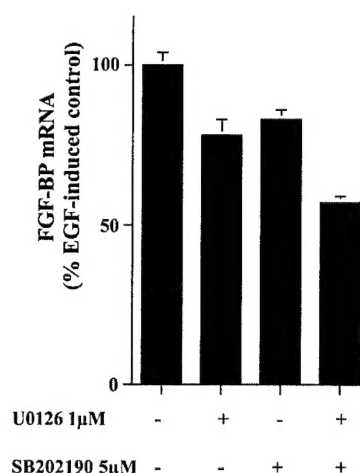

FIG. 7. **Additive contribution of ERK and p38 pathways.** FGF-BP mRNA levels from ME-180 cells treated with 5 ng/ml EGF for 6 h. Cells were pretreated for 1 h with vehicle alone or with suboptimal concentrations of U0126 (MEK1/2 inhibitor) and/or SB202190 (p38 inhibitor). Northern blot signal intensities of FGF-BP mRNA were quantitated, normalized to GAPDH, and expressed relative to mRNA levels after EGF treatment alone (without inhibitor), which was set to 100%. Basal FGF-BP level (without EGF or inhibitor) was approximately 25%. Values represent the mean and S.D. of at least two separate experiments.

duction of the FGF-BP promoter. In agreement with the dominant-negative data, expression of JNK had no significant effect on promoter activity, whereas expression of p38 consistently resulted in a higher level of EGF induction of FGF-BP transcription, which was significantly higher than the empty vector control (Fig. 8B). Furthermore, expression of a constitutively active mutant of MKK6, MKK6(Glu), a MAP kinase kinase that specifically phosphorylates p38 (25), was also tested for its effect on FGF-BP induction. Overexpression of MKK6(Glu) caused a significant increase in both basal and EGF-induced FGF-BP promoter activity (Fig. 8B). Overall, these data implicate ERK and p38 as being necessary for the full induction of FGF-BP by EGF. The lack of effect on FGF-BP promoter activity by either JNK overexpression or by inhibition of JNK through expression of a dominant-negative JNK mutant or expression of JIP, which prevents nuclear translocation of activated JNK (24) (Fig. 8B), suggests that JNK activation does not play a role in the regulation of FGF-BP in these cells.

DISCUSSION

This study shows for the first time that transcription of FGF-BP, an important mediator of FGF activation in SCC, is directly induced by EGF. The EGF family of growth factors, which include EGF, transforming growth factor-α, and other structurally related peptides, cause cellular signaling through the EGFR pathway, regulating proliferation and differentiation of many tissue types. Deregulation of the EGF-induced signaling network is known to play an important role in the tumorigenesis of several human cancers, including neoplasms of the brain, lung, breast, ovary, pancreas, prostate, and colon, as well as in SCC of the skin and cervix (11, 12, 34). Here, we have shown that EGF up-regulates FGF-BP gene expression, suggesting a link between activated EGF signaling in a cell and subsequent FGF activation, ultimately leading to activation of FGF-mediated processes, such as angiogenesis, during development and tumor growth.

The up-regulation of FGF-BP by EGF was found to show characteristics similar to TPA induction of this gene, including a rapid and transient increase in FGF-BP mRNA levels, and the requirement of transcriptional elements within the TPA
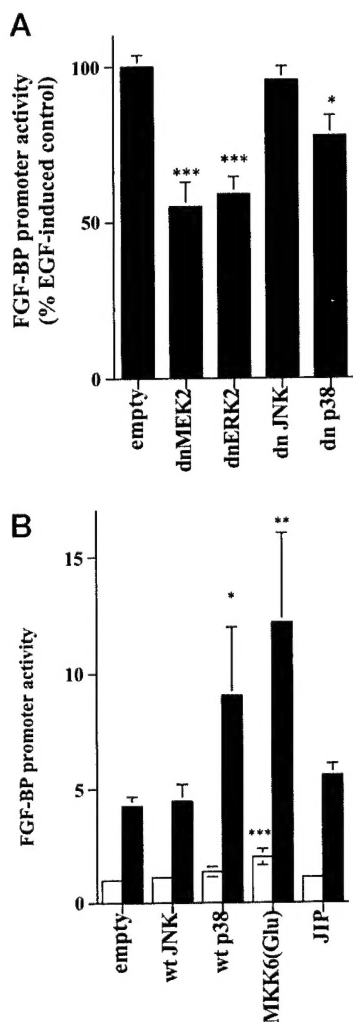
FIG. 8. **Effect of dominant negatives on EGF induction of FGF-BP promoter activity.** *A*, ME-180 cells were transiently co-transfected with the −118/+62 FGF-BP promoter construct along with either empty vector or with dominant negative mutant constructs for MEK2, ERK2, JNK, or p38. Transfected cells were either untreated or treated with 5 ng/ml EGF for 18 h. EGF induction is determined by the -fold increase in luciferase activity and expressed relative to the empty vector control, which is set at 100%. Basal (uninduced) promoter activity was approximately 18% of EGF-treated. Values represent the mean and S.E. from at least three separate experiments, each done in triplicate wells. Statistically significant differences relative to the empty vector control are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, *t* test). *B*, ME-180 cells co-transfected with −118/+62 FGF-BP promoter construct and either empty vector or expression vectors for JNK, p38, MKK6 (Glu), or JIP. Cells were treated as in *A*, and the FGF-BP promoter activity is shown relative to the untreated empty vector control. Statistical analysis was as described in *A*, and differences in the absence or presence of EGF are compared with the empty vector control in the absence or presence of EGF, respectively.

regulatory region (−118 to +62) of the promoter. Transcriptional regulation of FGF-BP by EGF required the AP-1 site between −65 and −61, which is bound by c-Fos and JunD family members (Figs. 2 and 3*B*). The finding that c-Fos is associated with FGF-BP up-regulation is significant, since an important role for c-Fos during SCC formation has been observed. Studies with c-*fos* knockout mice demonstrated that c-*fos* is required for malignant transformation of skin papillomas into malignant carcinomas, since c-*fos*(−/−) papillomas became desiccated and hyperkeratinized, lacked vascularization, and remained benign (35). Since the FGF-BP gene was previously found to be highly induced during skin carcinogenesis (8), it seems possible that this gene could be a target of c-Fos activation during skin SCC tumor formation, providing at least one mechanism for the recruitment of new blood vessels to the tumor.

The other required EGF response element in the FGF-BP promoter is the C/EBP site between −47 and −33, which is predominantly bound by the C/EBPβ and C/EBPδ family members (Figs. 2 and 3*C*). C/EBP proteins are a family of leucine zipper transcription factors that play a central role in the acute phase response and in a number of cell differentiation pathways (reviewed in Ref. 36). Regulation of C/EBP activity occurs at multiple levels, including increased gene expression (37, 38), nuclear localization (39), enhanced DNA binding (40), and post-transcriptional modification by protein kinases (27, 41). Because we observed no increase in C/EBP binding to the FGF-BP promoter after EGF treatment, it seems likely that stimulation of C/EBP transcriptional activity occurs through a post-translational modification. This conclusion is consistent with other studies demonstrating that TPA stimulation of the PKC pathway in hepatoma cells results in increased phosphorylation of C/EBPβ, enhanced transcriptional efficacy, and no obvious changes in DNA binding (27, 42).

C/EBP family members recognize similar DNA elements in their target genes, where they bind either as homodimers or heterodimers with other C/EBP family members or with other leucine zipper factors (43). Whereas C/EBPα is generally associated with growth arrest and cellular differentiation, C/EBPβ and C/EBPδ are often correlated with gene activation during cellular proliferation, inflammation, and tumorigenesis (44–46). Analysis of different C/EBP target gene promoters has demonstrated a finely tuned regulation of gene expression through the interplay of different C/EBP factors. During the acute phase response, for example, the amount of C/EBPα homodimers or heterodimers is reduced and replaced by C/EBPβ and C/EBPδ complexes (47–49). Interestingly, study of cyclooxygenase-2 promoter regulation by C/EBP during skin carcinogenesis showed a change in C/EBP complexes from C/EBPα·C/EBPβ in normal skin to predominantly C/EBPβ·C/EBPδ complexes in skin SCC (50). The presence of C/EBPβ·C/EBPδ complexes on the FGF-BP promoter in ME-180 SCC cells raises the possibility that FGF-BP up-regulation during tumor formation may be in part due to an interplay between the different C/EBP family members.

In trying to delineate the signal transduction pathway regulating FGF-BP gene expression, we found that activation of PKC plays a central role, since inhibition of PKC with calphostin C blocks both the EGF (Fig. 4) and TPA (10) induction of this gene. PKC activation in response to growth factor stimulus can lead to stimulation of the classical MAP kinase pathway Raf/MEK/ERK and subsequent activation of AP-1. PKC-mediated signaling through this pathway can be achieved through either Ras-dependent (15) or Ras-independent (16) mechanisms. The involvement of Ras in FGF-BP regulation is suspected, since FGF-BP expression is increased after DMBA treatment and in *ras*-transformed keratinocytes (8). A direct role for Ras in the activation of FGF-BP expression, however, has yet to be determined. In addition to PKC, the EGF-activated MEK/ERK pathway plays a significant role in the regulation of FGF-BP gene expression, since pharmacological and dominant-negative inhibition of MEK or ERK abrogates EGF induction. Although the exact target of ERK activation was not examined in this study, ERK has been shown to phosphorylate and activate both AP-1 (14) and C/EBP (41) family members.

p38 is a JNK-related MAP kinase that is activated in response to a variety of stimuli including growth factors, phorbol esters, cytokines, and environmental stress (17). Using a number of different approaches, we show in this study that in

addition to ERKs, p38 contributes to the EGF induction of FGF-BP. We demonstrated the inhibition of EGF-induced FGF-BP by pyridinyl imidazole compounds (SB202190 and SB203580), which selectively inhibit p38α and p38β2 isoforms but have no effect on the activity on other p38 isoforms, JNK, or ERK (31–33). In addition, we have shown that FGF-BP promoter activity is inhibited by expression of a dominant-negative p38 mutant but is activated by overexpression of wild-type p38. p38 is phosphorylated and activated by the dual specificity protein kinases MKK3, MKK4, and MKK6 (23, 25, 51–54). Although overexpression of the p38-specific kinase MKK6 can stimulate FGF-BP transcription, the signal transduction cascades that connect EGFR activation to phosphorylation of p38 in ME-180 cells remain unknown. One possible pathway that is currently under investigation is the involvement of two members of the Rho family of GTPases, Rac and CDC42, which are known to regulate the activity of both JNK and p38 (55–58). Furthermore, Rac and CDC42 can be activated downstream of Ras (55), thereby connecting p38 and JNK activation to growth factor effects on cell growth and proliferation. Transcription factor targets of p38 include CREB and ATF1 (59, 60), ATF2 (25, 61), MEF-2C (62), and the C/EBP family members CHOP (63) and C/EBPβ (64, 65), suggesting that C/EBPβ and/or C/EBPδ could be targets of p38 activation on the FGF-BP promoter.

In general, EGF regulation of FGF-BP gene expression seemed to be more dependent on the MEK/ERK pathway, since inhibition of MEK1/2 with U0126 completely abrogated induction, and expression of dominant negative MEK or ERK reduced induction by 50%. p38, on the other hand, appears to play a somewhat lesser role, since pharmacological inhibition of p38 caused a maximum 50% reduction of FGF-BP, and expression of dominant negative p38 reduced induction by only 23%. Although stimulation of the p38 pathway plays a less prominent role, regulation of FGF-BP by p38 may be independent of MEK/ERK activation, since they function in an additive rather than synergistic manner (Fig. 7). While the mechanism for these differences in activity of each pathway remains unclear, one explanation could be the differences in their transcription target specificities at the level of the FGF-BP promoter.

This study also examined the possible role of JNK in the regulation of the FGF-BP gene. Based on several lines of evidence, we have concluded that JNK is unlikely to be involved in FGF-BP regulation. Inhibition of JNK activity, either through expression of a dominant-negative JNK mutant or expression of JIP, had no effect on EGF induction of the FGF-BP promoter. Overexpression of wild-type JNK also had no effect on FGF-BP expression. In addition to this, we examined the activation of Elk1 by MEKK, a potent activator of JNK (66), and found no effect on this pathway by dominant negative JNK or JIP expression (data not shown). Furthermore, we found no activation of FGF-BP gene expression in the presence of UV light (data not shown), which is another known stimulator of the JNK pathway (17). Together, these findings indicated that there may be very little JNK activity in ME-180 cells and that JNK activation does not significantly contribute to FGF-BP gene expression in response to EGF.

In conclusion, this study demonstrates that the growth factor EGF induces FGF-BP gene transcription and characterizes the mechanisms by which this effect is accomplished. The EGF-mediated pathways leading to FGF-BP transcription and subsequent angiogenic activation include the selective activation of MEK/ERK and p38 MAP kinase pathways. This study highlights several targets for potential anti-angiogenic therapy of

human cancers, which utilize FGF-BP's angiogenic properties for tumor growth.

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